

INVESTIGATIONS OF A TWO-STEP PROCESS FOR POTATO (*Solanum tuberosum* L.) MICROTUBER PRODUCTION

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Biotechnology at the University of Canterbury.

Yoon, Kab Seog
2000

THE LORD is my sheperd, I shall not be in want. He makes me lie in green
pastures, He leads me quiet waters.
He restores my soul;
He guides me in paths of righteousness
for His name's sake.
Even though I walk
through the valley of the shadow of death,
I will fear no evil,
for You are with me;
Your rod and staff,
they comfort me.
You prepare a table before me
in the presence of my enemies.
You anoint my head with oil;
My cup overflows.
Surely goodness and love will follow me
all the day of my life,
and I will dwell in the house of the LORD forever. (PSALM 23:1-6)

ABSTRACT

Standard protocols for potato plantlet multiplication from nodal explants and for subsequent microtuberization were established. Liquid Murashige and Skoog (1962) basal media containing 3% (w/v) or 8% (w/v) sucrose without any exogenous plant growth regulators were used for plantlet multiplication or microtuberization respectively. More than 20 variations to the standard protocols, either during the plantlet multiplication step or the microtuberization step were investigated in relation to plantlet growth, microtuber number, average fresh microtuber weight and microtuber weight distribution. The responses of two potato cultivars ('Iwa' and 'Daeji') were compared. Time courses of some major changes in the media were also studied.

Initially, it was found that sucrose disappearance from the standard microtuberization medium, microtuber initiation, development and cessation of further growth, invertase activity development in the medium, osmotic potential changes and pH changes in the medium appeared to be correlative events. However, the data from the different experiments in this study indicate that most of these changes are associated with the 8% sucrose medium but are not strictly related to microtuberization.

Among the 21 variations to the standard protocols, whether during plantlet multiplication or during *in vitro* tuberization, medium replacement was most effective in inducing the formation of bigger and more microtubers.

In the course of this study, it was observed that at the end of the plantlet multiplication step the root had turned green. Even more interesting is that some of these green roots remained green after 10 weeks in darkness for the microtuberization step. A small-scale ultrastructural study confirms the occurrence of chloroplasts in the green roots during plantlet growth and also at the end of the microtuberization step in the dark.

ACKNOWLEDGEMENTS

I would like to thank Dr. David Leung for his endless supervision of this project. He always advised me when I felt my lackness of bioscientific knowledge, encouraged me when I was depressed of my living life and was joked me when I was sad because of my progress delayed.

Thank to Dr. Moon in Korea Institute of Machinery and Metals (KIMM) for his continued support over the distance. Dr. Yang in Korea Ginseng and Tobacco Research Institute, Dr. Joung in Korea Research Institute of Bioscience and Biotechnology, Dr. Seo in Chunnam National University and Dr. Bang in Iri National College of Agricultural Technology for their supports and discussions for potato microtubers commercialization were also appreciated.

My thanks to the staffs of the Plant and Microbial Science Department who have assisted me in various ways over the years, particularly Manfred for his TEM and Dougal and Matt for photograph, and technical assistances from Nicole and Vicki are gratefully acknowledged.

To all students, past and present in plant biotechnology laboratory, I am very grateful for your frendship and support, Kitti, Patalee, Martin, Simon, Suzan, Jason, Angela, Sincia. Visiting researchers Dr. Huh from Chunbuk National University and Dr. Eom from Kookmin University were also appreciated.

A very special thank to my family, particularly my wife Hee Je for continued support, encouragement and patience over my education and my whole life, and my son, Sang Chul and my daughter, Hye Mee, for their encouragement and support in domestic works.

Thank to my parents for their speechless support and long waiting and my brothers and sisters, Bo Hyun, Hyun Chul, Duk Hee and Myung Suk and also their families.

LIST OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGEMENTS	iv
LIST OF CONTENTS	v
LIST OF FIGURES	x
LIST OF TABLES	xiv
LIST OF PLATES	xv

I. Literature Review, Aim and Scope of this Research	1
1. Potato	1
1.1 Production of Potato	1
1.2 Diseases of Potato	2
1.3 Seed potato system	3
2. Micropropagation	4
2.1 <i>In vitro</i> potato culture techniques	4
2.1.1 Meristem, shoot-tip, and segment cultures	5
2.1.2 <i>In vitro</i> potato mass propagation	6
2.1.2.1 Multimeristem culture for micropropagation	7
2.1.2.2 <i>In vitro</i> shoot layering	7
3. Development of <i>in vitro</i> potato microtuberization	8
3.1 Use of <i>in vitro</i> tubers	9
3.2 Factors affecting <i>in vitro</i> potato microtuberization	11
3.2.1 Effect of phytohormonal growth inhibitors and growth retardants	12
3.2.1.1 coumarins	12
3.2.1.2 Chlorocholine chloride (CCC)	14
3.2.1.3 Absciscic acid (ABA)	14
3.2.1.4 Effect of TIBA (2,3,5-triiodobenzoic acid)	15
3.2.2 Effect of growth promoters	15
3.2.2.1 Cytokinin	15
3.2.2.2 2,4-D	17
3.2.2.3 Gibberellins	17
3.2.3 Environmental and other factors	18
3.2.3.1 Ethylene	18
3.2.3.2 Carbon dioxide	19
3.2.3.3 Nitrogen	20
3.2.3.4 Mineral ions	20
3.2.3.5 Activated charcoal in medium	21
3.2.4 Effect of growth conditions	21
3.2.4.1 Photoperiod and light quality	21
3.2.4.2 Temperature	23
3.2.4.3 Carbon sources and osmotic control	24
3.2.4.4 Liquid medium	26
4. Invertase activity	27

5. Maltose as a carbon source in liquid medium -----	29
6. Genotypic differences -----	32
7. Aim and Scope of this Research -----	34
II. MATERIALS & METHODS -----	36
1. Stock culture and propagation of plant materials -----	36
2. Systems for microtuberization -----	36
2.1 Bubbling jar culture -----	36
2.2 Shaking flask culture -----	36
2.3 Solid-liquid binary culture -----	37
2.4 Slanting jar culture and depth control (stationary culture) -----	37
3. Plant multiplication: basal or standard protocol -----	37
4. Changes during plantlet multiplication -----	38
5. Variations to the standard multiplication protocol -----	38
5.1 Different carbohydrate treatments -----	38
5.2 Replacement of the standard multiplication medium -----	38
6. Microtuberization: basal or standard protocol -----	39
6.1 time course of microtuber initiation and growth -----	39
7. Variations to the standard <i>in vitro</i> tuberization protocol -----	39
7.1 Varying concentration of sucrose in the tuberization medium -----	39
7.2 Retaining old multiplication medium on tuberization -----	39
7.3 pH treatments on tuberization -----	39
7.4 Replacing microtuberization medium -----	40
7.5 Osmotically equivalent media -----	40
7.6 Media with initial carbon content equivalent to that of the 8% sucrose solution -----	41
7.7 Medium containing maltose -----	41
8. Paper chromatography and HPLC analysis of sugars -----	41
9. Preparation of invertase and soluble protein from culture media -----	42
10. Partial purification of invertase -----	43
11. Extraction of crude invertase from potato plantlet tissues -----	43
12. Invertase assay -----	43
13. Amylase assay -----	44
14. Phosphatase assay -----	44
15. Protein quantification -----	44
16. SDS-PAGE -----	45
17. Non-denaturing PAGE and IEF -----	45
18. Cytochemical methods for localizing invertase activity in gel -----	46
18.1 Preparation of $[Ag(NH_3)_2]^+$ solution method - electrophoresis -----	46
18.3 The use of glucose oxidase, peroxidase and 3,3'-diaminobenzidine (D.A.B.) for detection of invertase activity following gel electrophoresis -----	46
19. Ultrastructural analysis -----	47
20. Osmotic potential measurements -----	48
21. Mineral analysis -----	48
22. Data analysis -----	48

III. RESULTS	49
A. Preliminary trials with different potato microtuberization systems	49
B. Main Experiments	54
1. Effect of manipulation of tuberization medium on microtuberization	54
1.1 Standard Protocol	54
1.1.1 Time course of microtuber development	54
1.1.2 Carbohydrate changes in the standard tuberization medium	54
1.1.3 Invertase activity in the standard tuberization medium	54
1.1.3.1 Preparation of invertase from the medium	54
1.1.3.2 Optimum pH of invertase activity	60
1.1.3.3 Change of invertase activity in the medium during tuberization	60
1.1.3.4 Presence of invertase in parts of stems and roots that were submerged in liquid medium	60
1.1.3.5 Invertase isozymes	60
1.1.3.6 Changes in soluble protein content during tuberization	64
1.1.4 SDS-PAGE of proteins in the standard tuberization medium	64
1.1.5 pH changes in the tuberization medium	64
1.1.6 Osmotic potential change in the standard tuberization medium	64
1.1.7 Time course of mineral changes in the standard tuberization medium	69
1.2 Effects of varying the concentration of sucrose in the tuberization medium	69
1.2.1 Effect on microtuber formation	73
1.2.2 Effect on soluble protein contents of tuberization media	73
1.3 Effect of different carbohydrates in the tuberization medium on tuberization	73
1.3.1 The initial osmolality of media containing different carbohydrates was equivalent to that of 8% sucrose (i.e the standard tuberization medium)	73
1.3.1.1 Microtuber formation	73
1.3.1.2 Osmotic potential changes of the different monosaccharide-containing media during microtuberization	79
1.3.1.3 pH of the media changed during microtuber formation	88
1.3.2 The effect of monosaccharide-containing media with carbon content that was initially equivalent to that of 8% sucrose	88
1.3.2.1 Microtuber formation	88
1.3.2.2 Osmotic potential changes in the different microtuberization media used in 1.3.2-	92
1.3.2.3 pH changes of the media used in 1.3.2 changed during microtuberization	99
1.3.3 Substitution of sucrose with maltose	99
1.3.3.1 Effect on microtuberization	99
1.3.3.2 Osmotic potential changes of the 8% maltose medium	104
1.3.3.3 Time course of pH changes in the 8% maltose medium	104
1.4 Effect of initial pH of tuberization medium	104
1.5 Effect of old multiplication medium mixed with tuberization medium	109
1.6 Effect of periodic refreshing of the standard tuberization medium	119

2. Manipulations during multiplication phase: effects on the during multiplication	127
2.1 Standard plantlet multiplication medium containing 3% sucrose	127
2.1.1 Plantlet development	127
2.1.2 Changes in the medium during potato development	127
2.1.2.1 Soluble proteins	127
2.1.2.2 Carbohydrates	127
2.1.2.3 Invertase activity in the medium	127
2.1.2.4 Change in the pH of the multiplication medium	134
2.1.2.5 Osmotic potential	134
2.2 Effects of variations to the standard multiplication medium	134
2.2.1 Different carbohydrate media for plantlet multiplication	134
2.2.1.1 Comparison of plantlet weights	134
2.2.1.2 Time course of soluble protein changes in different carbohydrate media ('Iwa')	143
2.2.1.3 Time course of carbohydrate changes	143
2.2.2 Effect of replacing medium during multiplication on growth of plantlets	143
2.2.3 Effect of manipulations during multiplication phase on microtuberization	148
2.2.3.1 Influence of carbohydrates in multiplication media on microtuberization under standard conditions	148
2.2.3.2 Effect of replacing treatment during multiplication on microtuberization	154
3. Ultrastructural Observations	159
3.1 Stem and leaf of <i>in vitro</i> potato plantlets ('Iwa') grown in liquid medium	159
3.2 Stem and leaf tissues submerged in liquid medium at the end of the standard multiplication step ('Iwa')	159
3.3.1 Stem and leaf tissues after microtuberization in liquid medium in the dark	168
3.3.2 Green roots in liquid tuberization medium	168
3.3.3 Microtubers in liquid medium	173
3.3.4 Floating cells in tuberization medium	173
IV. DISCUSSION	178
1. Is sucrose superior to glucose or fructose for potato microtuberization?	178
2. Can another disaccharide replace sucrose in the standard tuberization medium?	179
3. Invertase activity in culture medium	180
4. Relationship between osmotic potential changes in culture media and microtuberization and plant multiplication	183
5. Correlation between time course of carbohydrate changes and that of microtuber growth and plantlet growth	184
6. Possible interaction between carbohydrate levels in tuberization medium and gibberellin levels in stolon tips	185
7. Effect of medium-replacing treatments	186
8. Effect of sucrose concentrations and the Varying pH in the media on microtuberization	187
9. Ultrastructural studies	189

V. FUTURE STUDIES	191
VI. CONCLUSION	192
VII. REFERENCES	193
VIII. APPENDICES	209
1. Composition of Murashige and Skoog's Basal Medium	209
2. Estimation of glucose: HBH method	210
3. Protein concentration determination (Bradford's assay)	211
4. Recipes for SDS-PAGE	212
5. Silver stain procedure	215
6. Native isoelectric focusing gel	216
7. Desalting column preparation	217

List of Figures

Figure 1. Preliminary trials of 4 different microtuberization procedures -----	51
Figure 2. Time course of microtuber growth ('Iwa' and 'Daeji') -----	55
Figure 3a. Paper chromatographic analysis of carbohydrate changes in the standard microtuberization medium ('Iwa') -----	56
Figure 3b. Paper chromatographic analysis of carbohydrate changes in the standard microtuberization medium ('Daeji') -----	57
Figure 4a. Time course of carbohydrate changes in the standard tuberization medium ('Iwa') -----	58
Figure 4b. Time course of carbohydrate changes in the standard tuberization medium ('Daeji') -----	59
Figure 5. pH profile of invertase activity in standard microtuberization medium ('Iwa') -- -----	61
Figure 6. Time course of invertase activity development in the standard tuberization medium ('Iwa' and 'Daeji') -----	62
Figure 7. pH profiles of invertase activity in the extracts of 'Iwa' potato plantlets cultured in the standard tuberization medium. The root and stem parts that were submerged in the medium were used for enzyme extractions -----	63
Figure 8. Isozyme gel analysis of invertase -----	65
Figure 9. Time course of soluble protein changes in the standard tuberization medium ('Iwa') -----	66
Figure 10. SDS-PAGE of various liquid media -----	67
Figure 11. Time course of pH changes in the standard tuberization medium ('Iwa') -----	68
Figure 12. Time course of osmotic potential changes in the standard tuberization medium ('Iwa' and 'Daeji') -----	70
Figure 13. Time course of major inorganic ions changes in the medium during tuberization ('Iwa') -----	71
Figure 14. Effect of sucrose concentrations on average fresh microtuber weight ('Iwa') ---- -----	74
Figure 15. Effect of sucrose concentrations on average fresh microtuber weight ('Daeji') -- -----	75
Figure 16. Microtuber fresh weight distribution in response to different sucrose concentrations in the medium ('Iwa') -----	76
Figure 17. Microtuber fresh weight distribution in response to different sucrose concentrations in the medium ('Daeji') -----	77
Figure 18. Soluble protein changes in the media containing different sucrose concentrations during tuberization ('Iwa') -----	78
Figure 19. Effect of media osmotically equivalent to the 8% sucrose medium at the onset of the <i>in vitro</i> tuberization step on the number of microtubers formed ('Iwa') -----	80
Figure 20. Effect of media osmotically equivalent to the 8% sucrose medium at the onset of the <i>in vitro</i> tuberization step on the number of microtubers formed ('Daeji') -----	81
Figure 21. Average microtuber fresh weight in response to media osmotically equivalent	

to the 8% sucrose medium at beginning of the microtuberization step ('Iwa') -----82

- Figure 22. Average microtuber fresh weight in response to media osmotically equivalent to the 8% sucrose medium at beginning of the microtuberization step ('Daeji') -----83
- Figure 23. Fresh microtuber weight distribution in response to media osmotically equivalent to the 8% sucrose medium at beginning of the microtuberization step ('Iwa') -----84
- Figure 24. Fresh microtuber weight distribution in response to media osmotically equivalent to the 8% sucrose medium at beginning of the microtuberization step ('Daeji') -----85
- Figure 25. Osmotic potential changes of media that were osmotically equivalent to the 8% sucrose medium at beginning of the microtuberization step ('Iwa') -----86
- Figure 26. Osmotic potential changes of media that were osmotically equivalent to the 8% sucrose medium at beginning of the microtuberization step ('Daeji') -----87
- Figure 27. Time course of pH changes in monosaccharides-containing media that were osmotically equivalent to the 8% sucrose medium at the beginning of the microtuberization step ('Iwa') -----89
- Figure 28. Time course of pH changes in monosaccharides-containing media that were osmotically equivalent to the 8% sucrose medium at the beginning of the microtuberization step ('Daeji') -----90
- Figure 29. Average microtuber fresh weight in response to media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Iwa') -----93
- Figure 30. Average microtuber fresh weight in response to media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Daeji') -----94
- Figure 31. Microtuber fresh weight distribution in response to media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Iwa') -----95
- Figure 32. Microtuber fresh weight distribution in response to media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Daeji') -----96
- Figure 33. Osmotic potential changes of media with carbon contents that were equivalent to that of the 8% sucrose medium at beginning of microtuberization step ('Iwa') -----97
- Figure 34. Osmotic potential changes of media with carbon contents that were equivalent to that of the 8% sucrose medium at beginning of microtuberization step ('Daeji') -----98
- Figure 35. Time course of pH changes in monosaccharide-containing media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Iwa') -----100
- Figure 36. Time course of pH changes in monosaccharide-containing media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Daeji') -----101

Figure 37. Microtuber fresh weight distribution in response to media containing maltose or sucrose ('Iwa' and 'Daeji') -----	103
Figure 38. Osmotic potential changes of media containing maltose or sucrose ('Iwa') --	105
Figure 39. Osmotic potential changes of media containing maltose or sucrose ('Daeji') ----	106
Figure 40. Time course of pH changes in media containing maltose or sucrose ('Iwa') -----	107
Figure 41. Time course of pH changes in media containing maltose or sucrose ('Daeji') ---	108
Figure 42. Average fresh weight of microtuber in response to the initial pH of the tuberization medium ('Iwa') -----	110
Figure 43. Average fresh weight of microtuber in response to the initial pH of the tuberization medium ('Daeji') -----	111
Figure 44. Microtuber fresh weight distribution in response to the initial pH of the tuberization medium ('Iwa') -----	112
Figure 45. Microtuber fresh weight distribution in response to the initial pH of the tuberization medium ('Daeji') -----	113
Figure 46. Effect of mixing old multiplication medium with fresh tuberization medium on average microtuber fresh weight ('Iwa') -----	115
Figure 47. Effect of mixing old multiplication medium with fresh tuberization medium on average microtuber fresh weight ('Daeji') -----	116
Figure 48. Effect of mixing old multiplication medium with fresh tuberization medium on microtuber weight distribution ('Iwa') -----	117
Figure 49. Effect of mixing old multiplication medium with fresh tuberization medium on microtuber weight distribution ('Daeji') -----	118
Figure 50. Effect of medium replacement on the number of microtubers formed ('Iwa') ----	120
Figure 51. Effect of medium replacement on the number of microtubers formed ('Daeji') --	121
Figure 52. Effect of periodic medium replacement on average fresh weight of microtuber ('Iwa') -----	122
Figure 53. Effect of periodic medium replacement on average fresh weight of microtuber ('Daeji') -----	123
Figure 54. Effect of periodical medium replacement on fresh microtuber weight distribution ('Iwa') -----	124
Figure 55. Effect of periodical medium replacement on fresh microtuber weight distribution ('Daeji') -----	125
Figure 56. Longitudinal shaped microtuber (L) by periodic medium replacement treatment compared to a round shaped by the standard tuberization medium ('Iwa') -----	126
Figure 57. Time course of changes in the fresh and dry weights per plantlet during potato plantlet development ('Iwa') -----	128
Figure 58. Time course of changes in the fresh and dry weights per plantlet during potato plantlet development ('Daeji') -----	129
Figure 59. Time course of soluble protein changes in the standard plantlet multiplication	

medium ('Iwa') -----	130
Figure 60. Time course of carbohydrate changes in the standard plantlet multiplication medium ('Iwa') -----	131
Figure 61. Time course of carbohydrate changes in the standard plantlet multiplication medium ('Daeji') -----	132
Figure 62. pH profile of invertase activity in the plantlet multiplication medium ('Iwa') -----	133
Figure 63. Time course of invertase activity in the standard plantlet multiplication medium ('Iwa') -----	135
Figure 64. Time course of invertase activity in the standard plantlet multiplication medium ('Daeji') -----	136
Figure 65. Time course of pH changes in the standard plantlet multiplication medium ('Iwa') -----	137
Figure 66. Time course of pH changes in the standard plantlet multiplication medium ('Daeji') -----	138
Figure 67. Time course of osmotic potential changes in the standard plantlet multiplication medium ('Iwa') -----	139
Figure 68. Time course of osmotic potential changes in the standard plantlet multiplication medium ('Daeji') -----	140
Figure 69. Time course of plantlet growth in response to media containing different carbohydrates ('Iwa') -----	141
Figure 70. Time course of plantlet growth in response to media containing different carbohydrates ('Daeji') -----	142
Figure 71. Time course of soluble protein changes in multiplication media containing different carbohydrates ('Iwa') -----	144
Figure 72. Paper chromatography on time course of carbohydrate changes in the standard multiplication medium with maltose instead of sucrose ('Iwa') -----	145
Figure 73. Effect of medium replacement treatments on plantlet development ('Iwa') --	146
Figure 74. Effect of medium replacement treatments on plantlet development ('Daeji') ----	147
Figure 75. Effect of different plantlet multiplication media on average fresh weights of microtubers harvested at the end of the standard tuberization step ('Iwa') --	150
Figure 76. Effect of different plantlet multiplication media on average fresh weights of microtubers harvested at the end of the standard tuberization step ('Daeji') ----	151
Figure 77. Effect of different plantlet multiplication media on fresh weight distribution of microtubers harvested at the end of the standard microtuberization step ('Iwa') -----	152
Figure 78. Effect of different plantlet multiplication media on fresh weight distribution of microtubers harvested at the end of the standard microtuberization step ('Daeji') -----	153
Figure 79. Effect of medium replacement during multiplication on average fresh weight of microtubers formed at the end of the standard microtuberization step ('Iwa' and 'Daeji') -----	156
Figure 80. Effect of medium replacement during plantlet multiplication on fresh weight	

	distribution of microtubers formed at the end of the standard microtuberization step ('Iwa') -----	157
Figure 81. Effect of medium replacement during plantlet multiplication on fresh weight	distribution of microtubers formed at the end of a standard microtuberization step ('Daeji') -----	158

List of Tables

Table 1. Results of the preliminary trials in Fig. 1 at 10 weeks from the start of the
microtuberization step -----52

Table 2. Presence of enzyme activities in the medium at the end of the preliminary
tuberization experiments with ‘Iwa’ plantlets -----53

Table 3. Effect of varying sucrose concentrations on the number of microtubers formed
after 10 weeks in the dark -----72

Table 4. Effect of media containing carbon contents that were initially equivalent to that of
the 8% sucrose medium on the number of microtubers formed after 10 weeks in
the dark -----91

Table 5. Effect of maltose and sucrose on microtuber number (a) and average microtuber
weight (b) -----102

Table 6. Effect of old multiplication medium mixed with fresh tuberization medium

Table 7. Effect of different plantlet multiplication medium on the number of microtuber
formed in 8% sucrose tuberization medium (‘Iwa’ and ‘Daeji’) -----114

Table 8. Effect of medium replacement during plantlet multiplication on the number of
microtubers formed at the end of the standard microtuberization step (‘Iwa’ and
‘Daeji’) -----155

List of Plates

Plate 1. TEM view of chloroplast with small plasoglobulus in leaf of <i>in vitro</i> potato plantlet ('Iwa') grown in liquid medium -----	160
Plate 2. TEM view of mesophyll chloroplast in stem of <i>in vitro</i> potato plantlet ('Iwa') grown in white medium with thylakoid grana and a small plastoglobli -----	161
Plate 3. Typical chloroplast organization in the part of green stem that was submerged in the liquid plantlet multiplication medium -----	162
Plate 4. Grana and thylakoids in the stem tissue that was submerged in liquid medium at the end of the standard multiplication step -----	163
Plate 5. TEM view of mesophyll chloroplast in leaf tissue that was submerged in liquid medium fixed at the end of the standard multiplication step -----	165
Plate 6. TEM view of chloroplast with small plastoglobli in leaf tissue submerged in liquid medium at the end of the standard multiplication step -----	166
Plate 7a. Amyloplasts with large starch grains in stem tissue that was submerged after microtuberization in liquid medium in the dark -----	167
Plate 7b. Amyloplasts with large starch grains in leaf tissue that was submerged after microtuberization in liquid medium in the dark -----	168
Plate 8a. Chloroplast in cortical cells of photoautotrophic green roots -----	169
Plate 8b. Chloroplast in cortical cells of green roots -----	170
Plate 9. TEM view of a plasid of cortical cells from non-green root tissue in liquid tuberization medium -----	171
Plate 10. Typical TEM view of dictyosome of storage parenchyma cells with a few plastoglobli from non-green roots in liquid tuberization medium -----	172
Plate 11. Plastid of tuber cells which remained largely undifferentiated with a large starch grain in a non-greened microtuber in liquid medium -----	174
Plate 12. Mitochondria had cristae, and a matrix with a low density, in non-greened microtuber in liquid medium -----	175
Plate 13. Ovoid amyloplast of a non-green microtuber harvested at the end of the microtuberization step had a larger stromal volume with wide electron transparent zone and lamellae were more or less parallel to each other -----	176
Plate 14. Oval amyloplasts in cells floating in liquid microtuberization medium contained irregular shaped large starch grains -----	177

I. Literature Review, Aim and Scope of this Research

1. POTATO

The potato (*Solanum tuberosum* L.) belongs to the family Solanaceae. It is assumed that tuber-bearing *Solanum* species were first domesticated and eaten by man in the region of lake Titicaca in South America approximately 8,000 years ago (Hawkes 1978). The multitude of remote highland settlements in the vast Andean Cordillera, which stretches from Chile in the south to Venezuela in the north, provided numerous sites for selection and preservation of unique cultivated forms of potato. South Americans called the potato “batata”, whereas Spaniards later called it “patata”, from which the English name, potato probably originated. The Spanish were the first Europeans to discover this tuber crop when they invaded the Inca Empire in 1535. Around 1570 the crop was introduced to Spain and then to Ireland in 1590 (Bronk 1975). More than a century passed after these initial introductions to Europe before reports of widespread use of potato as food began to appear. Later, immigrants from Scotland and Ireland to the American colonies were the main conduits for potato introductions to North America.

1.1 Production of Potato

Potato is an annual crop plant, about 30-100cm tall and vegetatively propagated through tubers. The tuber bears the buds, commonly known as “eyes”, which sprout on germination and grow into plants.

The tubers, the size of which differs with age and cultivar, are grown in fields in ridges to maintain developing tubers under soil. The tubers start developing when the plant flowers, and their formation ceases when fruit formation begins.

The potato as one of the most valuable food crops is grown in more countries than any other crops except maize. Production volume of it ranks fourth in the world after rice, wheat, and maize. Potatoes accumulate the highest amount of energy per hectare per day during vegetative period, after sugarbeets, in tropical and subtropical countries. The potato tuber contains by weight, around 75-80% water, 16-20% carbohydrates, 2.5-3.2% crude protein, 1.2-2.2% protein, 0.8-1.2% minerals, 0.1-0.2% crude fat, 0.6% crude fiber, essential vitamins and trace elements (Singh and Verma 1979, Hooker 1983).

For human consumption, potatoes are used in fresh or in processed forms. Processing of potatoes for human consumption, already a major industry in the United States, is expected to continue expanding in both North America and Western Europe.

The potato is of highland origin, and in this traditional environment farmers generally cultivate no more than 1-2 ha in a number of distinct parcels of land, forming part of complex multiple cropping systems. Recently potato production in developing countries has spread gradually out of the traditional cultivation practice into commercially oriented large-scale farming. Intensive horticultural systems are more common where land is scarce and potatoes are expensive. In most areas, returns to potato growers are high on the average but highly variable. For this reason, few farmers specialize in potato production, and those who do tend to be large, financially well-off producers.

Seed tubers available to most developing-country farmers are costly and poor in quality. For these reasons, improvements in seed-tuber systems are needed to increase yields and reduce unit costs of production.

Research on true potato seed is also being conducted in developing countries. Early results indicate numerous potential applications in seed programs as well as directly by farmers growing table potatoes.

A wide range of techniques, including tissue culture and *in vitro* rapid multiplication, are currently being adopted to clean, maintain, and reproduce basic stocks for later multiplication and use by farmers. National programs are also experimenting with new institutional models for producing and distributing high-quality planting materials to farmers.

1.2. Diseases of potato

Different pathogens such as fungal, bacterial, viral, nematode, and abiotic diseases, affect different parts of the potato plant. Many have their origin in the preharvest period, but have effect on tubers before or after harvest. One serious disease in potato is the late blight caused by the fungus, *Phytophthora infestans*, which also caused the historic famine of 1845-1849 in Ireland. Similarly, many viral and wilt diseases are also common.

Knowledge of the occurrence and intensity of diseases and pests and the damage caused by

these is essential in order to take up preventive measures, and to develop disease-resistant variant methods. The motivating factor for potato improvement continues to be the development of late blight resistant cultivars.

1.3. Seed potato system

The conventional breeding of potato involves selection, crossing programs for recombination and mutation. Selection is limited to the variations detected so far. Moreover, it takes a long time to select new varieties and the efficiency of selection is limited. Starting with 100,000 seedlings it would take 6-8 years to obtain a better variety (Wenzel 1980).

Potato is propagated by tubers to preserve the parental properties and also because the seed setting is usually very poor. Even while propagating by tubers, sometimes variants appear which are called “bud sports”. Many variants do arise due to such vegetative mutations; the frequency of which is rather low. Mutations have been induced in potatoes by X-rays, UV and by chemical mutagens (Broertjes and Harten 1978). Practically all mutation work has been done using tetraploid cultivars and performed on tubers. Mutants have been obtained with success with regard to size, shape or colour of the tubers or leaves. Mutations for earliness, increased resistance to different diseases and increased starch content of tubers have been reported (Solomko 1965). Similarly, day neutrality was induced in several cultivars by chemical mutagens (Upadhy *et al.* 1974). However, in many cases mutations reveal themselves as periclinal chimeras, and hence show pleiotropic effects. Breeding for disease resistance is also attempted using crosses with *S.demissum*, which is resistant to many races of potato blight. First resistant gene R_1 and later R_2 was transferred to potato, but it is still not possible to impart resistance to all races of pathogen (Nelson 1984).

In order to make “normal” breeding methods possible, unconventional methods especially *in vitro* culture techniques, should be incorporated in potato-breeding schemes. One of the advantages would be to obtain “pure” monohaploid and dihaploid plants in large numbers by employing anther and pollen culture. These “haploid” plants can be diploidized again via the *in vitro* method to obtain homozygous lines, the availability of which would offer

the possibility of conventional crosses, and production of hybrid seeds, thus enabling the conversion of potato from a tuber-propagated crop to a seed-propagated crop. This would also have the advantage of producing virus-free plants, as tubers could carry many viral diseases, but the cost associated with the true potato seed appears to be too expensive.

2. Micropropagation

Mass plant propagation by tissue culture techniques, a facet of plant biotechnology, has developed into an important industry with considerable potential for the future (Vasil & Vasil 1986; Zimmerman *et al.* 1986). One of the key unresolved issues in mass propagation remains the high cost of producing plants from tissue culture.

Two important discoveries in the 1950s and early 1960s contributed to the development of modern methods for rapid mass propagation of plants *in vitro* or micropropagation. The first watershed was a report by Morel and Martin (1952) demonstrating that dahlia plants obtained from cultured shoot meristems were virus-free. This discovery led to the widespread culture of orchid meristems for plant propagation (Morel 1965; Morel 1960). The discovery of the role of cytokinins in shoot morphogenesis (Skoog & Miller 1957) was the second turning point. Today, most of the plant tissue culture propagation industry is based on culturing isolated shoot buds in media containing cytokinins. These plant hormones stimulate the development of multiple axillary buds by inhibiting apical dominance. Workers separate the newly formed buds by hand and reculture them to produce more buds. Eventually, buds are allowed to grow into shoots, which are then rooted in auxin-containing media to produce plants. Since each new plant arises from an organized meristem, the genetic fidelity of the variety is largely maintained.

This method has been used effectively to propagate a large number of herbaceous ornamentals, plus a few vegetables, fruits, and tree species. The entire process, however, is extremely labour-intensive and costly. It is impractical for large-volume, low-cost crops (potato, tomato, celery), or for plantation crops (rubber, coffee, tea, oilnut palm), and forest species.

2.1. *In vitro* potato culture techniques

2.1.1. Meristem, shoot-tip, and nodal segment cultures

Meristem and shoot tip cultures in general have been reviewed in detail by many researchers (Hu and Wang 1983; Kartha 1981; Quak 1979). Economical and technical aspects of *in vitro* tissue culture techniques have been widely reviewed (Levin and Vasil 1989). According to the article, tissue culture techniques would be economically feasible to get better quality of crop propagules such as potato, cassava, garlic, lilies, strawberries, carnation, pelargonium, gerbera, etc., which are susceptible to virus and other pathogens. Attempts have been made to increase the efficiency of the biological processes and to reduce the costs associated with the use of controlled environments for the growth and multiplication of cultures, and the acclimatization of plantlets in the greenhouse environment. *In vitro* meristem culture in potato is an established possible method of eliminating viruses (Bajaj and Sopory 1986; Wang and Hu 1985; Mellor and Stace-Smith 1977). *In vitro* meristem culture, sometimes combined with temperature or antiviral chemical treatments, is the only effective method to date that eliminates viral infections from systemically infected potato cultivars without inducing genetic changes (Wang and Hu 1980). Cultured meristem is also the preferred material for cryopreservation of germplasms (Kartha 1981, 1982). *In vitro* shoot tip and nodal segment culture of disease-tested potato material provides a rapid rate of clonal multiplication. With the aid of antiviral chemicals, shoot-tip culture is also capable of eradicating viral infections (Jordan *et al.* 1983). Unlike plantlet regeneration from callus culture, the meristem, shoot-tip, and nodal segment cultures provided genetic stability in the regenerated plantlets. According to Denton *et al.* (1977), potato plants regenerated from shoot-tip culture showed no differences in biochemical characters compared to the control plants. Meristem tip propagation was used by Wright (1983) to develop two or three virus-free clones of 10 cultivars. Field trials with these clones, which extended over 4 years, showed no consistent differences in yield or specific gravity of tubers among the clones of any of the 10 cultivars. Copeland (1982) compared potato nodal cuttings, consisting of a leaf attached to a piece of stem, with the traditional way of planting a seed tuber which gives a 6 to 10 fold annual multiplication rate, and it was suggested that thousands of plants may be

produced over winter from one tuber by micropropagating plantlets under controlled laboratory conditions. Nodal cuttings were kept at 25 °C under fluorescent lights for 16 hours each day. Nodal cuttings, which grew quickly and in 3 weeks had reached 5 cm in height, were ready by this time, for sub-division producing 3 to 4 more cuttings. Thus, similar results were obtained that the number of plants multiplied 3 to 4 fold using 10 varieties in every three weeks.

2.1.2. *In vitro* potato mass propagation

The importation of high cost certified seed tubers could become a major constraint to potato production. For example importation of certified seed tubers accounts for 40 to 50% of the total production costs of a potato crop in Indonesia (Wattimena 1983). In other countries such costs may reach 60% or more (Sawyer 1979). To overcome this problem, tropical or subtropical countries have to look for alternative methods of potato propagation that can be practiced locally and yet remain free of disease.

Micropropagation can be used to bulk up materials (1) after germplasm storage, (2) obtained from breeding-selection program, (3) generated by genetic engineering, or (4) for international quarantine-free distribution of propagated materials. Large-scale tests have been carried out to use *in vitro*-produced plantlets as the propagules of main crops (Wattimena *et al.* 1983). The Plant Resources Institute (Salt Lake City, Utah) has been testing commercial propagation of potatoes through tissue culture since 1980. They put 10 varieties encompassing 100 lines through the tissue culture system (Upham 1982). Six different micropropagation methods developed by various researchers for rapid *in vitro* cloning of potatoes have been described and compared (Goodwin 1982).

Micropropagated certified stock of potatoes can be transferred to the field in the form of rooted plantlets, *in vitro* produced microtubers, or *ex vitro* produced minitubers (produced from *in vitro* derived plantlets). Stem cuttings from *in vitro* produced plantlets are also used. Micropropagated seed stocks are reported to increase yield by 10-38% and show great uniformity (Jones 1988). Seed potato tubers production by micropropagation is expected to continue offering commercial opportunities in the following areas (Addy 1988): (1) Continued development of efficient micropropagation production systems that

in turn yield larger volumes of green house generated minitubers. This could reduce the field generation number necessary to supply commercial seed from indexed nuclear stock.

(2) Rapid introduction of new cultivars through large volume micropropagation / green house production systems. (3) Automated micropropagated systems that could increase the volume and decrease the unit cost possibly with the implementation of robotics in these systems. (4) Development of proprietary cultivars with rapid availability through efficient propagation systems.

2.1.2.1. Multimeristem culture for micropropagation

The culture method was developed at the International Potato Center in Lima, Peru (Roca *et al.* 1978). Rapid bud proliferation from excised shoot tips was enhanced by shake culture. Both axillary buds and adventitious buds were developed from the shoot tip explants and formed a multimeristem mass. The resulting shoots in turn were used to regenerate complete plantlets by nodal selection cultures. This is the most broadly tested (on 38 cultivars) of the potato micropropagation methods. Although callus-derived adventitious shoots were involved, no genetic changes were detected, using both biochemical and morphological criteria.

2.1.2.2. *In vitro* shoot layering

Shoot layering culture method was reported by Wang (1977) at the Academia Sinica, Taipei, Taiwan. Through repeated layering and subculturing of a single shoot, approximately 2,517 plantlets can be produced in 1 year. Large quantities of rooted shoots of several cultivars are thus produced annually, to be utilized as part of the process of virus-free potato production distributed to growers in Taiwan. Although growth regulator-free MS medium (Murashige and Skoog 1962) has been used satisfactorily in supporting repeated subculturing and development of nodal explants (Hussey and Stacey 1981), Wang (1977) found that the multiplication potential of axillary buds declined after 7 to 10 subcultures when no auxin was added. This loss in regeneration competence could be completely eliminated by enriching the medium with 0.005 μ M NAA. Both micropropagation methods described above included approximately this amount of NAA

in the multiplication medium. It was reported that after 56 days under an inductive environment the microtuber weight from layered shoots was 3-5 times greater than for nodal cuttings (Leclerc *et al.* 1994). Increased microtuber fresh weight from layered shoots compared with nodal cuttings may be due to increased production of endogenous growth regulators and gradients of these growth regulators. In addition, the increased leaf surface area available for the perception of the photoperiodic stimuli and surface area in contact with the medium may have contributed to the increased microtuber production. Favouring vegetative growth of stolon segments and shoots prior to microtuber induction has been reported to increase microtuber fresh weight (Chapman 1955; Garner and Blake 1989). They suggested that greater microtuber yield can be achieved by increased vegetative area of cultures prior to microtuber induction.

3. Development of *in vitro* potato microtuberization

Transplanting of tender vegetative plantlets from *in vitro* conditions to an external environment is rather laborious and has a high failure rate unless proper hardening procedures have been followed. On the other hand, dormant tubers could be harvested from *in vitro* conditions, stored, shipped, and planted rather conveniently (Roca *et al.* 1979; Ranalli *et al.* 1994a,b). Thus, it became advantageous to develop an *in vitro* mass tuberization method for potato propagation.

Many researchers have described techniques for mass propagation of microtubers (Estrada *et al.* 1986; Chandra *et al.* 1988) and its potential use for seed tuber production (Wang and Hu 1982; Wattimena *et al.* 1983). While microtubers generally originate as aerial structures on the stem, occasionally a few microtubers may be formed in the medium (Hussey and Stacey 1981, 1984). Akita and Takayama (1988a, b) reported a scale-up culture using jar fermentor techniques consisting of a 2 step culture by a method similar to the liquid shaken culture method reported by Estrada *et al.* (1986). Akita and Takayama (1988a, b) suggested the culture efficiency can be improved by multiplication of the shoots in the aerial phase and tuberization might be stimulated in the medium phase followed by continuous submersion of shoots. Akita and Takayama (1994b) reported shoots were cultured under the semi-continuous medium in which the medium surface level was raised

or lowered throughout the culture period. Tubers were induced and developed in every area in this jar fermentor system.

Akita and Takayama (1994a) first reported that potato microtubers can be propagated efficiently using an ebb-and-flow-type bioreactor (EFBR) system. Then Hulsher *et al.* (1996) reported that 1,600-1,700 potato tubers could be produced by using an EFBR system with a 10-liter culture vessel in 18 weeks (8 weeks for shoot multiplication and 10 weeks for tuber production). A similar type of culture system in which a slow rotating cylinder was employed has been used for mass propagation of potato microtubers. Microtubers were used as inoculants and multiplied further through induction of the microtuber shoot complex (Joyce and McCown 1991; McCown and Joyce 1991).

An *in vitro* mass tuberization procedure which was reported at the fourth International Congress of Plant Tissue Culture, has been used in seed potato production in Taiwan since 1975 (Wang 1978). Almost all the experiments carried out prior to 1978 involved only one to a few excised stolons cultured in each vessel for tuber initiation studies rather than the production of *in vitro* tubers in large quantity. Microtuberization was adopted at the International Potato Center (CIP) for germplasm storage and international distribution. CIP's international germplasm distribution plans for large volumes of potato were to gradually replace *in vitro* plantlets with *in vitro*-produced microtubers. The *in vitro* mass tuberization procedure was also adopted by McCown and co-workers at the University of Wisconsin in developing a complementary method of clonal propagation for commercial field planting (Wattimena *et al.* 1983). Improvements in the microtuber production method are still required since most current systems have problems obtaining sufficiently large microtubers for field planting (Ranalli *et al.* 1994) and a hormone-free medium is also desirable to avoid off-types in the progeny of microtubers as a result of culture-induced genetic variants.

3.1. Use of *in vitro* tubers

In vitro-derived storage organs are superior to shoots for micropropagation because they are easily acclimatized, stored and transferred. Easy storage and transport of the storage organs is also considerably advantageous in commercial production. On the other hand,

the efficiency of multiplication of storage organs is quite low compared with the propagation of shoots. As storage organs are induced from buds developed on shoots by modifying the physiological conditions, prolonged cultivation is usually required for the production of storage organs compared to the multiplication of shoots. In addition, only small numbers of buds usually develop into storage organs. So, applications of new techniques are now required to overcome the low propagation efficiency of storage organs. The practical use of *in vitro* tubers was realized in the early eighties with a view to producing disease-free seed potatoes for seed certification programs. *In vitro* tubers were used for international exchange of germplasm especially to those countries where the expertise for handling *in vitro* plantlets was not available. *In vitro* tubers were used to aid transport of germplasm under adverse conditions such as continuous darkness and variable temperatures, and as materials for medium-term conservation of potato germplasm. The use of *in vitro* tubers has been reported in China, Taiwan, Korea and Australia in the applied aspect of production of virus free potato seed.

Sluis and Rivera (1984) have reported that the *in vitro* tubers can be taken to the field as a source of disease-free seed stock.

In a seed potato production scheme in Taiwan 36,300 *in vitro* tubers could be produced in a period of four months in an area of 10 m², where 1,210 culture flasks of 500ml capacity were accommodated on the bench in the culture room (Wang and Hu 1982). These 36,000 “tuberlets”, after three successive field multiplications under disease-free conditions, produced 1,800 kg of seed potatoes which was sufficient for supplying seed potatoes for 2,000 hectares of land on a schedule of one third rotation per year. The yield from these virus-free clones was 26% higher than that of the farmer seed stocks. These are a few examples of application of this technology.

At the International Potato Center, Lima, Peru, this technique is being used for the international distribution of potato germplasm after pathogen indexing to different countries of the world. It is also being studied as an option for germplasm storage *in vitro* (Schilde *et al.* 1982; Estrada *et al.* 1986). Thime & Pett (1982) have reported the same use of *in vitro* tubers.

Many countries are using this technology in their potato production programs. The basic

techniques are already available in the literature and with a few modifications can be applied in many countries. Studies are underway in many laboratories to induce the maximum number of tubers per culture vessel and to increase mean tuber size. There are studies in Israel to attempt even mechanized production and harvest of *in vitro* tubers.

3.2. Factors affecting *in vitro* potato microtuberization

Barker (1953), who used etiolated sprouts to induce microtubers published the first report of *in vitro* tuberization by tissue culture methods in White's medium containing 80g/l sucrose. A similar and more extensive study had also been completed independently but concurrently (Mes and Mengo 1954). In a subsequent study, it was clearly demonstrated that microtubers would form from stolons that were free of bacteria and fungi (Barker and Page 1954).

Different aspects of *in vitro* tuberization have been studied. *In vitro* tuberization of potato has been studied by numerous workers (Wang and Hu 1982; Estrada *et al.* 1986). Work on microtuberization in potato has mainly focused on the use of growth regulators (Palmer and Smith 1969; Wang and Hu 1982; Estrada *et al.* 1986; Vecchio *et al.* 1994; Gopal *et al.* 1997; Gopal *et al.* 1998). It is well established that tuber formation in potato is regulated by carbohydrate and phytohormone fluxes (Ewing and Struik 1992; Vreugdenhil and helder 1992; Ewing 1995), and there is considerable variation in the results of these studies (i.e. the response obtained depended upon a range of factors including sucrose concentration, temperature, photoperiod, light intensity, and cultivar) (Hussey and Stacey 1984; Ortiz-Montiel and Losoya-Saldana 1987; Garner and Blake 1989). Gopal (1996) reported a faster rate of microtuberization and an earlier senescence of plantlets cultured under continuous darkness, the faster rate may have resulted in fewer eyes in microtubers. Then Gopal *et al.* (1997) described how the number of eyes affected performance for most characters, including tuber yield in a crop raised from microtubers under short days of low light intensity. Gopal *et al.* (1998) reported that short photoperiod (10 h of 6 -12 $\mu\text{mol}/\text{m}^2/\text{s}$) and low temperature (day 20 °C and night 18 °C) treatments had shown both higher yields than long day and (16h of 38 - 50 $\mu\text{mol}/\text{m}^2/\text{s}$) high temperature (day 28 °C and night 25 °C) treatments in 22 genotypes. Under short-day and low temperature

conditions the addition of BA increased microtuber yield and average microtuber weight. They also observed microtubers produced in the dark had a significantly lower number of eyes than those produced in light.

Since then the use of growth regulating materials to favor microtuberization has been the object of intensive investigation (Watimena 1983; Wang and Hu 1985; Chandra *et al.* 1988). However, growth regulators failed to induce tuberization when the sucrose supply was inadequate (Harmey *et al.* 1966). Sucrose appears to be the only carbon compound necessary for induction of microtuberization (Gregory 1956; Ewing 1985, 1990). Looking at the literature published so far it is evident that the physiological reactions of *in vitro* plantlets differ considerably from those of *in situ* plants, and also differ in many aspects from those of *in vivo* plants. Unexpectedly, conditions for *in vitro* tuberization, determined through numerous experiments, frequently do not coincide with *in situ* conditions. There are discrepancies among different publications as to which are the optimal levels for each of the factors involved.

The different factors included growth inhibitors and retardants (coumarin, ABA, CCC), growth promoters and regulators (cytokinins, auxins, ethylene), carbon source (sucrose, glucose, mannose) and growth conditions (temperature, photoperiod). But, a reliable production method involving a medium free of any growth regulating agents has also been reported (Garner and Blake 1989; Forti *et al.* 1991).

3.2.1. Effect of phytohormonal growth inhibitors and growth retardants

Compounds that inhibit vegetative growth frequently stimulate *in vitro* tuberization. Thus, many kinds of growth retardants and growth inhibitors have been used for *in vitro* tuberization studies. These compounds include ABA, CCC, MH, Alar, Amo-1681, and coumarin.

3.2.1.1. Coumarins

Coumarins are naturally occurring aromatic compounds which are found in a wide variety of plant species (Shah 1945; Kosuge and Conn 1959). Coumarin and its derivatives are known to have pronounced effects on physiological processes in both plants and animals.

While coumarin is considered to be primarily a plant growth inhibiting compound, researchers have also shown that coumarin can be a growth stimulant comparable to indole-3-acetic acid (IAA); however, it is considered to have a very different mode of action (Neumann, 1959; Neuman, 1960).

For the first time the effect of coumarin on *in vitro* tuberization of potato explants was reported in 1972 by Stallknecht. Different concentrations of coumarin were tried in the medium for tuber induction. In the medium used in this study no tuberization occurred at 1.0 mg/l coumarin or in the control lacking coumarin. However, at a concentration of 10 mg/l, 30% of the explants produced tubers. Coumarin at 25 to 50 mg/l was the optimum for the initiation of tubers. At 100 mg/l, the tuberization process was delayed, and the tubers were small compared to the treatments of 25 and 50 mg/l. Tubers formed in the coumarin-treated cultures were always larger in size than those grown in the medium with added kinetin (2.5 mg/l). Tuber initiation with kinetin treatment varied from 10 to 14 days, while the coumarin-treated shoots initiated tubers 2 to 3 days before. The time required for 100% tuberization varied from 15 to 20 days.

Stallknecht and Farnsworth (1982a) reported that high nitrogen, GA₃, ABA, IAA and NAA reduced the effectiveness of coumarin for the *in vitro* tuberization. GA₃ and high concentrations of nitrogen inhibit the uptake of coumarin by axillary shoots. The mode of action of these growth regulators on the inhibition of coumarin is not well known. The inhibitors of protein and nucleic acid synthesis i.e. chloramphenicol, para-fluorophenylalanine, actinomycin D and 5-fluorouracil were also evaluated for their effectiveness in inhibiting coumarin-induced tuberization. Chloramphenicol at concentrations from 25 to 100 mg/l almost completely inhibited tuberization. The amino acid analog para-fluorophenylalanine inhibited tuberization only at a concentration of 100 µg/ml. Actinomycin D reduced tuberization from 70% to 10% with increasing concentrations from 1.0 to 10 µg/ml. It also inhibited root growth and tuber diameter. 5-fluorouracil delayed tuberization at 1.0 and 5.0 µg/ml whereas at 10.0 µg/ml tuberization was reduced to 40%. However, it had no effect on any of the tuber characteristics (Stallknecht and Farnsworth 1982b).

Palmer and Smith (1970) reported that the inhibitors of protein and nucleic acid synthesis

(ACTD, PFA, 2-TU, CHL, 5-FUDR) delayed *in vitro* tuber formation but failed to inhibit the tuberization process when used in the medium.

3.2.1.2. Chlorocholine chloride (CCC)

Chlorocholine chloride (2-chlorethyl or trimethyl ammonium chloride) commonly called CCC or cycocel is a commercial growth retardant used in many crops. Its effect is variable in potato and it has been mainly used to induce tuberization under *in vivo* and *in vitro* conditions.

De Stecco and Tizio (1982) also studied the positive effect of CCC on *in vitro* tuberization of sprouts of potato cultured on basal medium without sucrose, while the controls did not tuberize at all.

Research at the International Potato Center, Lima, Peru has shown that CCC can easily induce *in vitro* tuberization. When included in MS nutrient medium at a concentration of 500 mg/l together with 5.0 mg/l BA and 8% sucrose, CCC is able to produce tubers in a broad range of genotypes in a period of 4 weeks (Schilde *et al.* 1982; Estrada *et al.* 1986). The GA₃-induced delay of *in vitro* tuberization can be overcome by the addition of CCC to the medium. Tizio and Goleniowski (1985) reported that CCC (1×10^{-1} M) inhibits the synthesis and/or release of GA₃-like substances from potato sprout sections cultured *in vitro*. This effect is closely linked with precocious tuberization of the explants. They further concluded that delayed root formation seems to be caused by a gibberellin or a complex of several gibberellins.

3.2.1.3. Absciscic acid (ABA)

Absciscic acid (ABA) is generally considered as a tuberization promoter, however, its role in tuber induction is a controversial matter. It appears that the role of ABA is primarily an indirect one and it acts by suppressing shoot growth, stimulating movement of carbohydrates to the tuber. ABA could play a role in cell and organ differentiation of tuberous roots by inhibiting root apical meristem activity and cell elongation (Melis and Van Staden, 1984).

Koda and Okazawa (1983) reported that ABA in the medium increased the tuberization

rate in combination with 2% sucrose. ABA, however, reduces or inhibits coumarin-induced tuberization (Stallknecht and Farnsworth, 1982a).

The inhibitor-complex and its probable main component, abscisic acid (ABA) is not the hypothetical tuber initiation factor in potato shoot sections cultured *in vitro*, nor does it seem to participate in the hormonal mechanisms proposed (Tizio and Maneschi, 1973). However, BA suppressed the effect of ABA on tuber induction (Kim 1982).

The effects of ABA on potato tuberization have been investigated and ABA is normally regarded as a regulator that reduces GA-promoted processes in plant development. It was assumed that ABA is a promoting hormone in potato tuberization (Okazawa & Chapman 1962; Marschener *et al.* 1984). However, the function of ABA with respect to stolon elongation, tuber initiation, and tuber growth is not clear. El-Antably *et al.* (1967) observed a stimulation of tuber formation by ABA applied to the leaves of long-day-grown potato plants. Wareing and Jennings (1980) found that ABA can replace the effect of the leaf by promoting tuberization in induced cuttings. The promoting effect of exogenous ABA was also demonstrated by the increasing numbers of tubers (Abdullah & Ahmad 1980), the earlier initiation of tubers, and the formation of sessile tubers (Menzel 1980).

Inhibition of tuberization by ABA was also reported with the effect depending on concentration and variety (Palmer & Smith 1969b; Hussey & Stacey 1984). The analysis of endogenous ABA showed an increase of ABA level under tuber-inducing conditions (Krauss & Marschner 1982) and a reduction of ABA content when N was supplied during tuber formation (Marschner *et al.* 1984). Further studies are needed to clarify the conflicting data concerning the effect of ABA on tuberization.

3.2.1.4. Effect of TIBA (2,3,5-triiodobenzoic acid)

TIBA (2,3,5-triiodobenzoic acid) has been reported by Lawrence and Barker (1963) to retard tuber formation but fails to prevent it. Its effect was not overcome by addition of IAA.

3.2.2. Effect of growth promoters

3.2.2.1. Cytokinin

The endogenous concentrations of different cytokinins vary in different parts of the potato plant (Palmer and Smith 1969, 1970; Smith and Palmer 1970). Forsline and Langille (1925) have confirmed the presence of four compounds with cytokinin-like activities in the potato plant and reported that their concentrations changed in proportion to variation of environmental conditions such as day length and temperature which have regulatory effects on the tuberization process. Courduroux (1967) suggested that a specific tuber forming substance may be related to cytokinin but he could not demonstrate a requirement for cytokinins in tuber induction.

Palmer and Smith (1969) reported for the first time that cytokinin is required in the tuberization of isolated stolons when cultured *in vitro* and showed an increase in the tuberization frequency on various cytokinin-containing media. 2.5mg/l kinetin (α -furfurylamino-purine) was more effective for tuber induction than other cytokinins. Kinetin and SD 8339 [6-benzylamino-9-2 (tetrahydropyran-2-yl)-9H purine] at all concentrations (0.25, 2.5 and 25.0mg/l) and N6 benzyladenine (BA) at 0.25 and 2.5mg/l induced 80-100% tuber formation in 90 days, although at 25 mg/l BA induced only 40% tuberization. No tuber initiation in cultures without cytokinin in the medium was observed. Their observation was supported by further investigations of exogenous cytokinins (Kumar & Wareing 1974; Hussey & Stacey 1984). The level of endogenous cytokinins was high in the induced tissue (Mauk & Langille 1978) and also during the later stage of tuber growth (Obata-Sasamoto & Suzuki 1979). However, exogenous cytokinins may also convert a stolon into a leaf-bearing shoot (Kumar & Wareing 1972). Smith and Palmer (1970) also reported that kinetin was effective in stimulating starch accumulation in excised potato stolons grown *in vitro*.

Furthermore kinetin (1.6×10^{-2} mM) induced 100% tuber formation when sucrose was present in the medium at 6 to 10%. However, sucrose without the presence of kinetin did not induce tuberization. Reduced temperature was partially inhibitory to the kinetin-induced response. A maximum of 50% tuber formation occurred on stolons incubated at

15°C for 30 days in the presence of kinetin only. The response to kinetin varied at different temperatures of incubation. Palmer and Barker (1972) also reported that kinetin at 2.0 µg/ml induced tuberization at 18°C in darkness. In the presence of kinetin, the rate of shoot elongation declined substantially after 7 days, which coincided with the onset of tuber initiation. In contrast, GA₃ treated, and control stolons continued to elongate throughout the period of incubation. Stallknecht (1972) reported that kinetin (2.5 mg/l) supplemented to the medium induced smaller tubers than coumarin induced tubers. Mingo-Castel *et al.* (1976) reported that 5 mg/l kinetin in the medium induces *in vitro* tuberization after 30 days in culture with 94% efficiency compared to 61% in the absence of kinetin. The sucrose concentration was 6% in both cases. Kim (1982) stated that 2.5 mg/l BA in the medium induced tuberization, whereas, Wang and Hu (1982) reported that 10 mg/l BA in the medium with 8% sucrose induced *in vitro* tubers in total darkness giving an average of 30 to 35 tubers per flask of 500 ml. 2.0 mg/l BA and 6% sucrose in the medium was found to be optimal for tuberization under short days (8 hours light). Zeatin did not stimulate *in vitro* tuberization even when its concentration was high (10⁻⁵ M). However, it induced an increase in fresh weight of tubers when combined with high sucrose concentration i.e. above 4% (Koda and Okazawa 1983). The use of 10 mg/l BA, 100 mg/l chlormequat or 1 mg/l Alar 85 (active constituent diminozide) in the medium stimulated tuber development in 10 varieties tested for *in vitro* tuberization (Kostrica *et al.* 1985).

3.2.2.2. 2,4-D

Mangat *et al.* (1984) had reported the effect of 2,4-D on *in vitro* tuberization. After 15 days, the percentage of tuberization was highest at the lower concentration of 2,4-D (i.e. 10⁻⁷ and 10⁻⁶ M) in comparison to the control (no 2,4-D). Individual tuber size was greater in cultures without 2,4-D and at higher concentrations of 2,4-D long stolons rather than tubers were observed.

3.2.2.3. Gibberellins

Gibberellins (GAs) are cyclic, diterpenoid hormones with an essential role in plant growth

and development. They control a variety of growth responses in higher plants, including stem elongation, fruit set, flower induction, seed germination, and mobilization of seed reserves (Hooley 1994; Swain & Olszewski 1996). Gibberellins are growth hormones that affect growth and dry matter distribution in the potato tubers and have been studied widely. Most investigators agreed that the application of gibberellins in different forms results in shoot growth (Menzel 1980). In potato (*Solanum tuberosum*), exogenous application of GAs has a strong inhibitory effect on tuberization (Okazawa 1960; Tizio 1971). Harmey *et al.* (1966) had reported that GA₃ inhibits *in vitro* tuberization while IAA and maleic hydrazide (MH) stimulates it. The application of gibberellin inhibitors also has the reverse effect on tuberization (Bodlaender 1964; Kumar and Wareing 1974).

Bottini *et al.* (1981) reported that an increased level of endogenous GA₃ was found in CCC treated plants and that GA₃ delays tuberization *in vitro*. The sprouts cultured in a medium containing 1 ppm GA₃ did not tuberize at all. On the contrary they formed thin and highly branched stoloniferous shoots which is a typical effect of this phytohormone.

Gibberellins are known to prevent tuber formation, even when other conditions are tuber-inducing as in the presence of high sucrose being one of the inducing factors in the tuberization medium (Koda and Okazawa 1983; Vreugdenhil and Struik 1989).

Tuber formation in potato is promoted by short photoperiods, cool temperatures, and low rates of nitrogen fertilizer (Ewing 1990). GA activity was shown to decrease when leaves were exposed to short days (Pont-Lezica 1970; Kumar & Wareing 1974), with as few as two short days being sufficient to cause a decline in the GA activity of *S. tuberosum* subsp. *andigena* leaves (Railton & Wareing 1973). It was shown that changes in GA activity occurred not only in response to photoperiod, but also to the other environmental conditions that affect tuberization. (a) High temperatures increased GA activity in buds (Menzel 1983). (b) A continuous supply of nitrate in the hydroponic solution increased GA activity in shoots and prevented tuberization (Krauss & Marschner 1982). (c) A decrease in GA₁ level was observed when the stolon tips started to swell in *in vitro*-cultured single-node cuttings grown in a high-sucrose tuber-inducing medium (Xu *et al.* 1998). Furthermore, tuberization was improved by the application of inhibitors of GA synthesis such as paclobutrizol or ancimydol (Menzel 1980; Hussey & Stacey 1984;

Ewing 1995; Jackson & Prat 1996).

3.2.3. Environmental and other factors

3.2.3.1. Ethylene

The role of ethylene on tuberization of potato has been a controversial issue. Some scientists believe it induces tuberization while others feel it inhibits the tuberization process. Bodlaender (1964) showed that the effect of ethrel depended very much on the concentrations tested.

The tuberization process involves inhibition of axial growth and increase in radial expansion of the tuber (Jolivet, 1959). Garcia-Torres and Gomez-Compo (1973) were the first scientists who studied the effects of ethrel on *in vitro* tuberization of etiolated potato sprouts, which was considerably advanced by a dose of 50 ppm ethrel. Ethrel also increased the number of tubers, produced shorter and thicker stolons and reduced root development. Some of these effects are opposite to those induced by gibberellic acid, which is known to retard tuberization and promote elongation. When both substances were simultaneously supplied to the medium they showed a clear antagonistic interaction. The effect was reduced stolon elongation, increased stolon thickness and reduced root development.

Palmer and Barker (1973) published a contradictory report on the action of ethylene. The response of potato stolons, cultured *in vitro*, when ethylene was applied at 0.067, 0.67 and 67 μ M was that no tuber formation resulted, as in the control. They also reported that ethylene influenced stolon growth but this may not be directly related to tuber initiation.

3.2.3.2. Carbon dioxide

Mingo-Castel *et al.* (1974) reported the findings that CO₂ stimulated tuberization of isolated potato stolons cultured *in vitro*. The stimulatory effect of CO₂ is inhibited by ethylene, which is by itself also inhibitory to tuberization. There is an antagonistic action of CO₂ and ethylene on tuberization *in vitro* but CO₂ and kinetin that was required for tuberization were both inhibited by ethylene. CO₂ could not overcome the ethylene inhibition. Ethylene totally prevented root formation and development, inhibited stolon

elongation, and caused thickening and diageotropic growth of the stolon. In addition, ethylene prevented the accumulation of both starch and red anthocyanin, which are often present in the tuber. Three to five days of exposure to CO₂ were required to obtain induction of tuberization of stolons cultured *in vitro*. Bicarbonate ions did not alter starch synthetase activity isolated from potato tubers *in vitro*. Koda and Okazawa (1983) reported that gibberellin inhibited tuberization while the application of either ethrel, (2-chloroethyl phosphonic acid) or 1-aminocyclopropane-1-carboxylic acid caused diageotropic growth habit of the lateral shoot, but failed to induce tuberization at any of the concentrations tried.

Hussey and Stacey (1984) found that the restriction of gas exchange inhibited tuberization under a wide range of conditions and that ethylene accumulation appeared to be the major factor in *in vitro* tuber initiation and growth inhibition.

3.2.3.3. Nitrogen

In general, a medium containing high nitrogen is inhibitory to *in vitro* tuberization, especially the coumarin-induced ones. Compounds containing reduced nitrogen also inhibit tuberization. Stallknecht and Farnsworth (1979) and Wattimena (1983) found that low nitrogen (2.5mM) in both the explant and the tuberization media was best for coumarin-induced tuberization. No tubers were formed if high nitrogen (60mM) was present in both explant and media. The inhibitory effect of nitrogen can be partially relieved by using a high concentration of sucrose (Okazawa 1967). Stallknecht and Farnsworth (1979) found that the inhibition of coumarin-induced tuberization at intermediate levels of nitrogen could be overcome by adding 12% sucrose but not 6% sucrose. Cytokinin-induced tuberization was not sensitive to the inhibitory effect of a high nitrogen level in the medium. Whether reduction in nitrogen level in the medium will further improve cytokinin-induced *in vitro* tuberization rate has been tested (Palmer and Smith 1969; Wang and Hu 1982).

3.2.3.4. Mineral ions

The importance of the chemical environment in multiplication and microtuber induction *in*

vitro has been demonstrated for potato. Mineral composition as a chemical environment is an important component of tissue culture media. There are few reports on the effect of mineral nutrition during microtuberization. It has been shown that mineral nutrition together with nitrogen strongly influenced the development of potato microtubers *in vitro* (Sarkar and Naik 1998). It is, however, difficult to explain the differential effect of mineral nutrition in association with inorganic nitrogen on microtuberization. Processes associated with supplying minerals to plantlets *in vitro* have recently been investigated (Williams 1992, 1993, 1995). An extension of this work in the present thesis is to clarify the effects of minerals in microtuberization *per se* rather than in growth of plantlets.

3.2.3.5. Activated charcoal in medium

The presence of activated charcoal in nutrient media promotes cell and tissue culture by the adsorption of various inhibitory metabolites or other substances released during growth (Fridborg *et al.* 1978; Drew 1979).

It was suggested that charcoal probably affects the nitrogen biochemistry and ammonium:nitrate ratio inside the plant, for it has been shown that nitrogen nutrition strongly influences the development of microtubers (Garner and Blake 1989). Also, the first stage in the sequence of anatomical events in tuber initiation is the change from longitudinal to radial cell enlargement (Booth 1963), followed by radial cell division (Plaisted 1957). The possibility that activated charcoal interacts in these stages cannot be ruled out.

Bizarri *et al.* (1995) reported the effects of activated charcoal in hormone-free MS medium supplemented with sucrose (8%, w/v) compared to medium containing growth regulators (cytokinins, polyamine biosynthesis inhibitor and chlorocholine chloride) used for microtuber induction and development. The results showed that the cultivars exhibited a wide range of variations in the mean weight of microtubers, and nearly all plants produced tubers. Medium containing activated charcoal gave the highest rate of tuberization and the largest microtubers. Thus, they suggested that activated charcoal (0.2%, w/v) played a role in optimizing conditions for rapid, mass tuberization, and produced large microtubers for field planting.

3.2.4. Effect of growth conditions

3.2.4.1. Photoperiod and light quality

Light requirements for *in vitro* tuberization varied among different reports. Some used continuous light, or alternating light, others alternating light and darkness with continuous darkness. In general, at 20 to 25°C, darkness, or continuous low light intensity (<500 Lux) is known as the most effective for *in vitro* tuberization.

Barker (1953) for the first time described a comparison on induction of tubers in the dark and under continuous illumination. Lawrence and Barker (1963) found that their culture produced tubers only under continuous darkness or continuous darkness interrupted with a low light intensity. Wang and Hu (1982) reported that a low light intensity of 100 lux for 8 hours is the optimum for *in vitro* tuberization. Schilde (1982) found that microtuber production was faster when plantlets were induced to tuberize in the dark rather than an 8-h photoperiod. Stallknecht and Farnsworth (1982) also produced microtubers under darkness. Thieme and Pett (1982) observed *in vitro* tuberization when the photoperiod was less than 12 h. Sattelmacher and Marschner (1978) and Mares *et al.* (1981) selected a 12-h photoperiod to carry out their tuberization research. Hussey and Stacey (1981) observed that microtubers were formed in cultures incubated for 16- and 24-h photoperiods, but not an 8-h photoperiod. Wattimena (1983) used 0, 8, or 24 h of light per day and concluded that the longer the photoperiod, the better the tuberization. But almost all the experimenters induced tubers in the dark for their research (Claver 1956, 1967, 1977; Stallknecht 1972; Palmer and Smith 1969, 1970; Mingo-Castel *et al.* 1974, 1976; Schilde *et al.* 1982, 1984; Estrada *et al.* 1986; Koda and Okazawa 1983; Mangat *et al.* 1984). Hussey and Stacey (1984) obtained results further supporting the general view that darkness is better for *in vitro* tuberization.

An intensity of 800 lux was used by Hussey and Stacey (1981). A slightly lower intensity was used by Wattimena (1983). Wang and Hu (1982) used a very low intensity of 100 lux and found that 35.4 microtubers were produced in each culture flask at this intensity, compared to 27.3 and 13, at 1 and 4 kilolux respectively. The above discrepancies may have partially resulted from whether or not cytokinin was used in the culture medium by various workers. It seems that a longer photoperiod with higher light intensity is required

when cytokinin is not used. The reason for such a light requirement is still obscure. In 1959, Makronosov *et al.* showed that a night break of 20 min in the middle of a dark period of 14h inhibited tuber formation in *Solanum tuberosum* L. cv. Larch and in *Solanum demissum*. Much later, phytochrome involvement in this response was confirmed by showing reversibility of the inhibitory red light break effect by subsequent far-red irradiation (Batitus and Ewing 1982). Recently *Solanum tuberosum* ssp. *Andigena* expressing the potato *PHYB* cDNA displayed a typical response of photoperiodic regulation of tuber formation (Jackson *et al.* 1996).

Wild species of *Solanum*, such as *S. demissum* or *S. tuberosum* ssp. *Andigena*, represent an ideal model for studies of photoperiodic control of both flowering and tuber formation. In long days (LD) they flower and in short days (SD) they form tubers (Konstantinova *et al.* 1991). Phytohormones are one of the signals that could take part in transducing the effects of photoperiod on growth and morphogenesis, as was demonstrated in the case of tuber formation using night break as an experimental tool (Machácková *et al.* 1998). Plants grown under night break displayed many growth characteristics intermediate between SD and LD. Under LD and night break regimes ABA levels in all organs were about one-fourth of those under SD. An opposite trend was found for gibberellin content: it was very low in SD-grown plants and 4-10 times higher under both other conditions with the exception of roots and stolons in night break. The levels of free indoleacetic acid and cytokinin were high in LD- and night break-grown plants and much lower in SD-grown plants, including tubers.

3.2.4.2. Temperature

Palmer and Smith (1970) found that at a temperature of 15°C no tuber induction was achieved and at 35°C complete inhibition of tuberization occurred. Okazawa (1967) found that temperature below 12°C was strongly inhibitory. Temperature over 28°C became inhibitory. According to Hussey and Stacey (1981), the optimal temperature range was 20-25°C with slightly faster tuberization at 20°C. However, Thieme and Pett (1982) obtained their *in vitro* tubers at 8 to 10°C, and Wattimena (1983) detected only slight reduction in tuber formation at 10°C compared to the optimal temperature of 15°C. Most workers

chose 20°C (Sattelmacher and Marschner 1978; Mares *et al.* 1981) or 25°C (Stallknecht and Farnsworth 1982; Mingo-Castel *et al.* 1974, 1976, 1976) for their *in vitro* tuberization studies. Nevertheless the optimal temperature determined by Wattimena (1983) was 15°C. He suggested the reason for the low temperature becoming the tuber-inducing agent was that the high intensity of light might have resulted in a higher actual temperature inside the culture vessels.

3.2.4.3. Carbon sources and osmotic control

The carbon source in the medium is a very important factor for *in vitro* tuberization. Sucrose concentrations in the medium used vary from 2% to 12% in different reports. Gregory (1956), working with one-node stem pieces from induced and non-induced Kennebec potato plants, reported tubers from induced materials on 3% sucrose in a solid medium beginning after 4 days. The non-induced materials tuberized within 25 days on 3-10% sucrose. Okazawa (1955) found no tubers from stem cuttings on solid media with 2-4% sucrose. However, tubers formed when the level was raised to 6-10%. Gregory (1965) concluded that sugars are necessary as energy sources for enlarging tubers. He doubted that sugar is the direct determining factor for tuber initiation, a view shared by Lawrence and Barker (1963). Tuber formation is maximal at 8-12% sucrose (W/V) on modified White's (1943) solid medium (WS) dropping off sharply at 16% (Lawrence and Barker 1963). Palmer and Smith (1969) used only 2% sucrose in the medium and indicated that the cytokinin concentration affected *in vitro* tuberization even at low sucrose concentration. The same results were also reported by Palmer and Barker (1972), whereas Claver (1977) obtained tubers using 2% sucrose in the medium without any additives. Koda and Okazawa (1983) used 4% sucrose in the medium in combination with cytokinins.

Mingo-Castel *et al.* (1976), Hussey and Stacey (1981, 1984), Palmer and Smith (1970) have used 6% sucrose in the medium but they also used kinetin.

Other workers have used 8% sucrose in the medium (Wang & Hu 1982; Schilde *et al.* 1984; Estrada *et al.* 1986) with growth regulators, particularly, kinetin or coumarin for *in vitro* tuberization while Lawrence and Barker (1963) used 8% sucrose alone in the

medium and obtained tubers. Fung *et al.* (1972) reported that as the level of carbon source increases the *in vitro* tuberization process is speeded up. They state that the osmotic concentration of the medium is not the crucial factor for tuber initiation and growth, but have not speculated on a specific role for carbohydrate in tuberization.

Fung *et al.* (1972) cultured etiolated stolon nodes on White's medium to which sucrose was added at 2, 8 or 12%. The osmotic potential of the media was kept the same using different concentrations of mannitol. Rapid tuber induction was observed on cultures growing on 8 and 12% sucrose, but none on media to which only mannitol was added. Only a few small tubers were formed after a great delay on low sucrose media, regardless of the presence of mannitol. Thus, they concluded that microtuber induction was in response to the high sucrose concentration, and was independent of the osmotic potential of the medium.

The effect on *in vitro* tuberization of different carbon sources such as sucrose, glucose, fructose, mannose and mannitol at concentrations ranging from 4 to 12% have been studied in two potato clones. Sucrose at a concentration of 8% was found to be optimal for good tuber size and number. Glucose and fructose at all the concentrations studied yielded tubers of smaller size than sucrose. Mannose and mannitol failed to induce tuberization by Tovar and Dodds (1987). Garner (1987) attempted a similar experiment, when he used mannitol to raise the osmolarity of Murashige and Skoog (1962) solid medium with 4% sucrose to that of MS medium with 8% sucrose. He found that on media containing mannitol microtuber induction was delayed by 4 weeks, and by the end of the experiment (14 weeks), only a few microtubers had developed on that medium, compared to around twice as many on 4% sucrose alone, and around four times as many on 8% sucrose. It was assumed from these results that microtuber induction was inhibited by mannitol addition. Oparka and Wright (1988) showed that sucrose uptake and its conversion to starch in potato tuber discs was sensitive to the osmotic potential of the medium and of cells themselves. They found that starch synthesis was optimal at 300mM mannitol, a value remarkably close to the measured tissue osmolarity, but decreased sharply above and below this. Garner and Blake (1989) showed that the effect of 8% sucrose in solid MS medium compared to 4% was to increase microtuber number and fresh weight. Khuri and

Moorby (1995) tested media containing sucrose, maltose, glucose or fructose supplemented with 4% sucrose media, while keeping the osmotic potential of the media constant. A concentration of about 400mM of sucrose in the medium was more suitable for microtuber production than media supplemented with maltose, glucose or fructose. When glucose was supplied at concentrations that had the same number of carbon atoms as 8% sucrose, the high osmolarity inhibited microtuberization. They concluded that sucrose acts as a suitable carbon source for uptake and utilization by the plantlets, but, at 8%, it also provides a favourable osmolarity for the development of microtubers.

3.2.4.4. Liquid medium

It is increasingly evident that the concentration and type of matrix used in nutrient media can profoundly influence the response of tissue cultured *in vitro* (Ziv 1991). All large-scale plant propagation systems currently use semi-solid agar or similar media for the multiplication phase of shoot buds (Aitken-Christie 1991, Levin *et al.* 1988). Excessive liquid or humidity in culture vessels causes a condition known as vitrification (hyperhydricity), in which cell walls fill with water and suffer metabolic and morphological abnormalities (Schloupf *et al.* 1995). Because vitrification leads to severe growth reduction, if not death, it has been accepted as dogma that shooty cultures can neither be maintained nor multiplied in submerged liquid cultures. Traditionally, liquid cultures, whether in small flasks or in large bioreactors, have been used only for the growth of highly dispersed non-morphogenic cell lines of embryogenic cultures. There is now a proprietary procedure that prevents vitrification and allows growth of a great variety of species in liquid media (Ziv 1991).

Growth in liquid medium offers the following advantages:

- (1) Growth as well as handling of the cultures is simpler and more efficient. This enables saving in space, time, and labor.
- (2) Accurate monitoring and control of temperature, pH, carbon dioxide, oxygen, and ethylene in the bioreactors provide more uniform growth conditions.
- (3) Contamination is minimized, since the automated bioreactor and the bioprocessor are integrated and maintained in a single, closed, sterile system. The plant material is

neither handled directly nor exposed to a non-sterile environment during incubation and transfer.

- (4) Production in a large-scale bioreactor is more easily automated than it is in small, individual culture vessels. It is also easier to automatically transfer cultures, or nutrient media, in and out of a large vessel than from many individual flasks.

Work has been done on the advance of process developments of a large scale propagation by meristem and shoot-tip culture (Escalada & Garcia 1982; Goodwin *et al.* 1980; Roca *et al.* 1978; Henshaw & Roca 1976; Morel 1975; Roca 1975; Murashige 1974) and proliferation by axillary shoots developed from *in vitro* cultured nodal cuttings (Espinoza *et al.* 1984; Hussey & Stacey 1981; Roca *et al.* 1978; Nozaran *et al.* 1977). It has been shown that the important factor influencing microtuberization is a multiplication step to optimize *in vitro* shoot growth to obtain high quality potato seed tubers (Bryan *et al.*, 1981; Copeland 1982). Considerable research has been done on the nutritional (Wetherell and Dougall 1976; Dougall 1981; Avila *et al.* 1994) and hormonal (Singha 1982) aspects of culture media preparation; however, the physical state of the culture media and its effects on the mechanisms that regulate explant growth have received less attention (Singha 1982). It has been observed that liquid or low-agar concentration media increase the growth of certain species cultivated *in vitro* (Singha 1982; Debergh 1983; Rossel *et al.* 1987). Low-agar concentration media increased root and shoot growth of apple plantlets cultivated *in vitro* (Romberger and Tabor 1971; Singha 1982). Liquid media increased the growth of cv. Spunta two-fold (Rossel *et al.* 1987), but up to now the growth mechanisms have not been elaborated upon. Previous findings indicate that the improved growth in species cultivated in liquid or low-agar concentration media is caused by greater availability of water (Bouniols, 1974; Debergh, 1983) and nutrients (Singha 1982; Debergh 1983). This availability may be induced by a lower resistance to diffusion of materials released from plant cells and closer contact between the explant and the culture medium (Romberger & Tabor 1971; Hammerschlag 1982; Singha 1982; Pierik 1990). However these effects have not been fully proven yet.

4. Invertase activity

Plant tissues are either net exporters of carbohydrate (source organs) or net importers (sink organs). Various types of sink organs range from those that are metabolically highly active, such as meristems and young developing organs, to storage organs such as fruits, seeds, and tubers. The factors governing the distribution of photoassimilate between the various types of sink organs is a central question in plant physiology, particularly given the importance of sink organ productivity for agriculture. The capacity of a sink to draw in and metabolize photoassimilates seems in competition with the other sink organ.

In the majority of agriculturally important plants sucrose is the end product of photosynthesis in source organs, and is the form in which carbohydrate is distributed through the sieve element systems to sink organs. The initial cleavage of sucrose in sink organs proceeds either by invertase or sucrose synthase. In potato tubers, the vast majority of sucrose degradation is catalyzed by sucrose synthase and invertases play only a modest role, if any.

Invertase (β -D-fructofuranosidase, E.C.3.2.1.26) is a highly polymorphic glycoprotein that cleaves sucrose into hexose sugars (Myrback 1960). Investigation of plant tissues has revealed more than one activity of invertase on the basis of pH optima, isoelectric points, solubility characteristics and intra or extra-cellular localization. In plants, invertases can be separated on the basis of pH optima into two groups, acid and alkaline. The soluble alkaline invertase is thought to reside in the cytoplasm (Ricardo and ap Rees 1970). Two types of acid invertase have been observed: a soluble (vacuolar) and a particulate form (apoplasmic or cell-bound). In dicotyledenous plants these compartment-specific isoforms are encoded by separate genes. The role of acid invertases in plants is complex and may change in response to developmental (Strum *et al.* 1995) and environmental signals such as wounding (Matsushita and Uritani 1974; Strum and Chrispeels 1990), gravitropism (Wu *et al.* 1993) and pathogen infection (Strum and Chrispeels 1990; Tang *et al.* 1996). Acid invertases are considered to be important in growth, providing tissues with hexoses as a source of energy and carbon. In developing seeds of fava bean (*Vicia fava*) apoplasmic invertase in the seed coat is thought to result in a high concentration of hexoses which are taken up by the developing embryo (Weber *et al.* 1995). In some plants root apoplasmic

invertase is thought to facilitate phloem unloading by maintaining a steep sucrose gradient between source (photosynthetic cells) and sink (heterotrophic cells) regions of the plant (Eschrich 1980; Morris and Arthur 1985). In other plants, such as carrot tap roots, where phloem unloading occurs at least partially through membrane-located pumps (Daie 1984; Hole and Dearman 1994), sucrose synthase, located in the cytoplasm, and vacuolar acid invertase are considered to be more important in generating and maintaining the sucrose gradient between source and sink tissue (Strum *et al.* 1995). Recently, vacuolar invertase activity has been reduced in the fruit of tomato using antisense technology in order to examine the role of soluble acid invertase in determining the sugar composition of tomato fruit (Ohyama *et al.* 1995). Red ripening tomatoes accumulate hexose as a result of hydrolysis of sucrose by either intracellular invertase or sucrose synthase. In transgenic tomato fruit where invertase activity was low sucrose content was markedly elevated when compared to wild-type fruit, clearly showing that the high activity of acid invertase in non-transgenic/normal ripening fruit prevents the accumulation of sucrose. An antisense approach has also been used to reduce acid invertase activity in potato tubers (Zrenner *et al.* 1996). In these plant vacuolar invertase appeared to control the hexose:sucrose ratio in cold-store tubers.

In order to investigate the importance of the sucrose cleavage step for sink strength, Sonnewald *et al.* (1997) expressed yeast invertase in transgenic potato tubers using a tuber-specific promoter. Although there is strong evidence that the unloading of sucrose from the phloem into tuber storage-parenchyma cells proceeds by direct cell to cell transfer through plasmodesmata (symplastic unloading), there is still a possibility that some sucrose moves into parenchyma cells via the extracellular space (apoplastic unloading) (Oparka *et al.* 1992). Sonnewald *et al.* (1997) therefore decided to construct two transgenic potato lines, one where the invertase was expressed in the cytosol and a second where the invertase was targeted to the apoplast of potato tubers. The yeast invertase has previously been expressed in the cytosol and apoplast of potato, tobacco, and *Arabidopsis* plants using the 35S promoter to confer constitutive expression (von Shaewen *et al.* 1990; Sonnewald *et al.* 1991; Büsis *et al.* 1997). Sonnewald *et al.* (1997) showed that expression of the invertase in the cytosol led to a reduced tuber size and increased

tuber number per plant, whereas localization in the apoplast resulted in a marked increase in tuber size and a decrease in tuber number. So, they suggested sink organ size could be manipulated through modification of sucrose metabolism.

5. Maltose as a carbon source in liquid medium

For *in vitro* culture of plant tissues, sucrose is generally regarded as the standard carbohydrate component of culture media (Evans *et al.* 1981; Dunwell 1985). However, for the development of embryos and green plants from anthers, microspores and cultured cells of a number of plant species, maltose has been shown to be better than sucrose, glucose or fructose. Embryogenesis from anthers of *Solanum tuberosum* L. (Batty and Dunwell 1989), *Triticum aestivum* L. (Last and Bretell 1990; Orshinky *et al.* 1990), *Hordeum vulgare* L. (Hunter 1988), and somatic embryogenesis from *Brassica napus* L. and *Brassica oleracea* L. protoplasts (Loudon *et al.* 1989) and *Medicago sativa* L. calli (Strickland *et al.* 1987) are some examples where maltose instead of sucrose, glucose, or fructose in the culture medium led to an increase in the number of embryos or plants regenerated from cell cultures. These observations are remarkable since sucrose is the dominant source of energy and carbon supplied to non-photosynthesizing plant tissues (Hawker 1985), whereas maltose plays only a minor role in plant carbohydrate metabolism. Thus, sucrose would be expected to be a better carbohydrate source than maltose for embryogenesis from anthers, and growth of calli. Furthermore, when sucrose is supplied to non-photosynthetic plant tissues, it is frequently hydrolyzed by cell wall invertases to glucose and fructose before entering the cells in which it is metabolized (Lucas and Madore 1988). Hence, glucose and fructose would also be good carbohydrate sources for supporting growth of cultured plant cells. In contrast, maltose is not known to be supplied to non-photosynthetic plant cells; therefore, it would not be a better carbohydrate source than sucrose, glucose or fructose for the culture of plant tissues. The different effects of sucrose, glucose, and maltose upon embryogenesis have been studied in greater details in barley (Sovari and Schider 1987; Hunter 1988; Finnie *et al.* 1989). Hence, barley is the best species to investigate further the different effects of these sugars upon embryogenesis. However, the majority of the work on barley has been with

anthers, which are made up of a number of different tissues, such as microspores, phloem tissue and the tapetum cell layer. Since microspores can be readily isolated from barley anthers experiments on cultured microspores should be ideal for clarifying these previous observations. Only limited work has been performed with barley microspore culture (Hunter 1988), but the available results suggest that maltose is the better carbohydrate source than other sugars for embryogenesis from microspores, as it is for anther culture. In *Abies alba*, maltose was found superior to sucrose and glucose in terms of formation of mature embryos (Hristoforoglu *et al.* 1992). Early experiments with *Abies normanniana* had also indicated that maltose was superior to sucrose (Nørgaard and Krogstrup 1991, Nørgaard 1992) but quantitative documentation was insufficient. Scott and Lyne (1994) showed the effect of maltose using isolated microspores of *Hordeum vulgare* L. cv. Igri in the presence of different sugars. Incubation of microspores in the presence of sucrose, glucose or fructose resulted in the death of cells via some unknown toxic mechanism. In contrast to this, maltose can sustain development of embryoids and calli from cultured microspores. The only two enzymes known to metabolize maltose in plants are α -glucosidase and maltose phosphorylase. The former yields glucose and the latter produces glucose and glucose 1-phosphate. Finnie *et al.* (1989) suggested that the rate at which sucrose was metabolized was the cause of the above observations but no data were available for that hypothesis. Scott *et al.* (1995) found that cultures on sucrose differed from those on maltose in that there was a significant accumulation of ethanol and a lower adenylate energy charge. It was suggested microspores cultured on 40 mM sucrose die because they metabolize the sugar rapidly, become hypoxic and, as a result, accumulate a large quantity of ethanol within the cells. Metabolism of maltose is slower and there is sufficient oxygen available to allow cells to survive in culture.

Nørgaard (1997) confirmed maltose was superior to sucrose in terms of the number of mature somatic embryos of the conifer *Abies nordmanniana* formed as well as their germination percentage. Optimum maltose concentrations varied between 3.4 and 4.3%, but mixtures of glucose and maltose did not show as good results as pure maltose.

Khuri and Moorby (1995) tested media containing sucrose, maltose, glucose or fructose supplemented with 4% sucrose media, while keeping the osmotic potential of the media

constant. A medium concentration of about 400 mM with only sucrose was more suitable for microtuber production than media supplemented with maltose, glucose or fructose.

When maltose was supplied at concentrations, which had the same number of carbon atoms as 8% sucrose, microtuberization was not inhibited. Although they concluded that sucrose at 8% acts as a suitable carbon source for uptake and utilization by the plantlets, the same concentration of maltose also provides a favourable carbon source for the development of microtubers.

In their experiments, the effect of maltose was not clear because all the media they tested were supplemented with 4% sucrose, so the effect seems to be produced by a mixture of sucrose and maltose not maltose alone.

6. Genotypic Differences

The discrepancies between the findings from papers are probably due at least in part to the use of different genotypes. Mostly, the genotypes exhibited a wide variation in harvest index in response to applied treatments.

The effect of liquid and solid media on the growth of micropropagated potato shoots (cvs. 'Spunta', 'Kennebec' and 'Huinkul') was studied in relation to the availability of nutrients and water in the culture medium (Avila *et al.* 1996). Despite of the difference in cultivar, similar trends were observed in that all the plantlets showed marked increases in elongation and dry weight in liquid rather than in solid medium. The effects of activated charcoal on induction and development of microtubers in potato (*Solanum tuberosum* L. cvs. 'Monalisa', 'Primura' and 'Spunta') were studied by Bizzari *et al.* (1995). The cultivars showed wide variations in the mean weight of microtubers, ranging from 44.6mg (cv. 'Primura') to 77.5mg (cv. 'Spunta'), and nearly all plants produced tubers. But they concluded that medium containing activated charcoal gave the highest rate of tuberization and largest microtubers. The qualitative and quantitative aspects of the production of potato microtubers (*Solanum tuberosum* L.) were also studied in a two-phase system (Defernandez *et al.* 1995). Nodal cuttings of cvs. 'Spunta' and 'Bintje' were grown on solid MS medium and then liquid tuberization medium added on top of it. The results showed that the two-phase (overlaying the 60ml of solid propagation MS medium with

45ml of modified MS tuberization liquid medium) optimized the microtubers' weight (769.5mg for cv. 'Spunta' and 498.1mg for cv. 'Bintje') and decreased the percentages of abnormalities for both cultivars.

Rapid induction of microtubers was observed in the micropropagated, layered potato shoots of cvs. 'Kennebec', 'Russet Burbank' and 'Superior' in medium devoid of growth regulators (Leclerc *et al.* 1994). However, the microtuber weight per shoot was significantly influenced by cultivar, culture type, and growth regulator and by both cultivar-culture type and cultivar-growth regulator interactions. Layered shoots of cvs. 'Russet Burbank' and 'Superior' produced significantly greater microtuber weight and number per shoot than cv. 'Kennebec'.

The effect of the use of growth retardants to improve microtuber formation on potato (*Solanum tuberosum* L. cvs. 'Arran Banner', 'Cara', 'Caribe' and 'Spunta') was studied by Harvey *et al.* (1991). They showed that 50% of the nodal segments cultures of cv. 'Desiree' tuberized in the absence of chloromequat and this was increased to 100% by inclusion of 10^{-3} M chloromequat in the medium whereas all nodal segments of cv. 'Cara' tuberized in the absence of chloromequat and its addition reduced the percentage of cultures that tuberized.

The induction of *in vitro* tubers in a broad range of potato genotypes was reported by Estrada *et al.* (1986). It was shown that the formation of *in vitro* tubers was induced in over 50 different genotypes possibly.

7. Aim and Scope of this Research

From the literature review, there does not seem to be a shortage of findings concerning the factors that influence at least some aspects of microtuberization. It is particularly notable that many studies took advantage of isolated potato stolons as a simple experimental system for basic investigations into microtuberization. In these studies, quantitative data about aspects of microtuberization in relation to effects of plant growth regulators or other factors could usually be obtained rather conveniently.

From the applied research point of view, a two-step process such as that described by Leclerc *et al.* (1994) and several other researchers is more appropriate for further research. This comprises first growing *in vitro* plantlets from nodal explants and then introducing microtuber formation on the plantlets, both steps being in liquid media without the need for any exogenous plant growth regulators.

Research is needed to improve this process so that it will be a practical, efficient and cost-effective means to mass produce microtubers that are big enough to compete with field-produced seed potatoes for planting out. The long-term aim is for automation of this process.

The goal of the present research is to fill in some knowledge gaps in a two-step practical process towards the production of more and larger microtubers. One main problem emerging from the literature review is that it is not clear whether the findings on tuberizing isolated stolons can be applied meaningfully to a more complex two-step tuberization process. Also, our understanding of the microtuberization process is incomplete due to a lack of precise information on the changes of the medium. This might seem insignificant as it would be expected that at some point of *in vitro* culture the medium components will be depleted. Nevertheless, a time course study of changes in sucrose in the medium, for example, might shed some new light on the relationship between carbohydrate supply and timing of microtuber initiation, microtuber growth and timing of cessation of microtuber development, and may even yield new experimental opportunities to optimize the microtuberization process further. At present, it is not clear whether sucrose in the medium

is required for all these aspects or only certain aspects of the microtuberization process. However, it is particularly important to determine precisely the relationship between sucrose status in the medium and, for example, cessation of microtuber development. If sucrose depletion is correlated with cessation of microtuber growth, it would then be interesting to see the outcomes of refreshing the medium at predetermined time points.

Finally, most published studies on a two-step process of microtuber formation are complicated by not studying the effects of experimental manipulations or medium changes with due regard to the distinct nature of the two steps involved. For instance, in the study of Khuri and Moorby (1995), the nodal explants were placed right from the beginning of a two-step process on a medium containing 8% sucrose, which is known to induce microtuberization. Not only could there be potential problems for plantlet growth in this initially high concentration of sucrose, but also it is not possible to determine how much direct influence the medium had on the different aspects of microtuberization. This and associated problems have been addressed in this research.

II. MATERIALS AND METHODS

1. Stock culture and Propagation of Plant Materials

In vitro potato stock plants (*Solanum tuberosum* L. cv. Iwa and cv. Daeji) were regularly propagated from nodal cuttings every 6-8 weeks on MS basal medium (Murashige and Skoog 1962) supplemented with 30g/l (w/v) sucrose and solidified with 7g/l (w/v) agar. All media in this work, unless indicated otherwise, were autoclaved (20 min., 121°C) after the pH was adjusted to 5.8. A single nodal segment from a previously propagated stock plant culture was placed in a clear polycarbonate jar (64mm IDx80mm, total capacity of 250 ml) containing 40ml of the medium. All cultures were incubated at 23°C under continuous irradiation of 60 $\mu\text{mol}/\text{m}^2/\text{s}$ at the top of the vessels from fluorescent lamps (Philips).

2. Systems for microtuberization

2.1 Bubbling Jar Culture

Step 1: Multiplication

A 'home-made' screw-capped jar fermentor (total capacity of 1 liter) with sintered glass bubbler was used in a 2-step procedure for microtuberization. Twenty segments of single nodes excised from stock plants of 'Iwa' were transferred into the jar fermentor containing 200 ml of full-strength MS basal medium and 30 g/l sucrose without plant growth regulators. Explants were cultured with continuous sterile air supply (flow rate, 200 ml/min through 0.48 μM Millipore cellulose acetate membrane filter) at 25 °C for 4 weeks under continuous light.

Step 2: Microtuberization

The medium was drained and refilled with 600 ml of growth regulator-free MS basal medium containing 80 g/l sucrose. Jars were aerated with sterile air (flow rate, 200 ml/min) at 20 °C in the dark.

2.2 Shaking flask culture

Aliquots of 20ml of the MS basal medium supplemented with 3% sucrose were dispensed

into 100 ml conical flasks. After autoclaving, three excised shoot tips from stock plant cultures of 'Iwa' were placed in each culture vessel. Culture vessels after inoculation with explants were left stationary for 3-4 days, and then they were placed on a reciprocal shaker having 30 oscillations per minute in a culture room at 25 °C with continuous light for 4 weeks. For microtuberization, the medium was drained and refilled with 40 ml of the MS basal medium supplemented with 8% sucrose. The cultures were kept on a reciprocal shaker having 30 oscillations per minute at 20 °C in complete darkness for 10 weeks.

2.3 Solid-liquid binary culture

Solid culture was performed as described for stock plant culture propagation for the first 4 weeks in the light at 25 °C before 50ml of MS liquid medium containing 8% sucrose were added for microtuberization. This binary culture system was kept at 20 °C for 10 weeks in complete darkness.

2.4 Slanting jar culture and Depth control (Stationary culture)

Three shoot segments each with 2 nodes that were excised from the stock plants were transplanted into clear polycarbonate jars (64 mm IDx80 mm), each containing 20 ml of the MS basal medium with 4% sucrose. After shoot generation within 2 weeks, the jars were slanted by about 15 degree to submerge the bottom part of the plantlets into liquid medium up to 4 weeks under continuous light. Then the medium was drained off and refilled with 50 ml of the MS basal medium containing 8% sucrose. The cultures were then incubated at 20 °C in darkness for 10 weeks.

3. Plant multiplication: Basal or Standard protocol

Three stem segments, each comprising 2 nodes, were excised from the previously propagated stock plant culture and were transferred to the same type of polycarbonate jars containing 25 ml MS liquid culture medium. This medium comprising full strength MS hormone-free medium was supplemented with 30 g/l sucrose. The pH of the medium was adjusted to 6.0 before autoclaving at 121 °C for 20min.

Cultures were kept in a growth room at 23 °C, illuminated with fluorescent tubes (Philips) giving an average irradiation of 60 $\mu\text{mol}/\text{m}^2/\text{s}$ at the top of the vessels. Explants were cultured for 4 weeks before the tuberization phase began.

4. Changes during plantlet multiplication

For the time course experiments, all the plant materials in polycarbonate jars were harvested weekly and their fresh weights were recorded. Then the dry weights of the plantlets were obtained after 24 h in an oven at 80 °C. Media samples (20ml) were also collected and stored at 4 °C for monitoring the changes in carbohydrates, pH and invertase activity. Three independent determinations were carried out to get the average value.

5. Variations to the standard multiplication protocol

5.1 Different carbohydrate treatments

MS basal medium was supplemented with or without, 30 g/l sucrose or 30 g/l maltose. The sugar solutions were filtered through 0.22 μM membrane filters (type GS, Millipore Corporation, U.S.A.) before being added to the rest of the autoclaved liquid MS medium for shoot multiplication. After 4 weeks of the multiplication phase, the plantlets were subjected to the standard tuberization protocol for 10 weeks. The effect of the two variations, sugar-free and maltose, compared to the standard protocol (3% sucrose) on changes during multiplication as well as possible effects on the number of microtubers formed, and their weight distribution were studied.

5.2 Replacement of the standard multiplication medium

The effect of replacing the medium during multiplication on the subsequent microtuberization process was evaluated in relation to average microtuber weight, number of microtubers formed, microtuber weight distribution and carbohydrate changes during multiplication. In this experiment, after 2 weeks of culture the standard multiplication medium was drained off and replaced by 25 ml of fresh standard multiplication medium as per the protocol. One week later, the medium was again replaced. Seven days later, the plantlets were transferred to the standard microtuberization protocol.

6. Microtuberization: Basal or Standard protocol

After 4 weeks of the plantlet multiplication phase, the residual medium was drained off and replaced by 50ml of microtuberization medium at pH 6.0 consisting of full-strength MS basal salt and organics solution containing no growth regulators, supplemented with 80 g/l sucrose for tuberization. All the cultures were incubated at 20 °C in darkness for 10 weeks.

6.1 Time course of microtuber initiation and growth

For the time course experiments, microtubers were harvested weekly, and fresh weight of individual microtubers was measured. The media were also collected weekly for measurement of pH, soluble protein contents, invertase activity, osmotic potential and carbohydrate content.

7. Variations to the standard *in vitro* tuberization protocol

7.1 Varying concentration of sucrose in the tuberization medium

The variation involved addition of different concentrations of sucrose ranging from 2%-20% to the MS basal medium. Microtubers were harvested 10 weeks following the transfer of the plantlets from the multiplication medium to these tuberization media. The harvested fresh microtubers were weighed individually.

7.2 Retaining old multiplication medium on tuberization

The effect of adding microtuberization media containing different concentrations of sucrose at the end of 4 weeks of plantlet multiplication without first discarding the original multiplication medium was studied. The cultures were harvested after 10 weeks following the mixing of old multiplication medium with the appropriate tuberization medium.

7.3 pH variations in tuberization medium

Two variations to the pH of the standard tuberization medium, which was 6.0, were attempted. Before autoclaving the media, the pH of the otherwise standard tuberization

medium was adjusted to 4.0 (the low pH variation) or 8.0 (the high pH variation). The pH of the autoclaved media changed: the low pH medium rose to about pH 4.3 and the high pH medium dropped to about 7.8. After 4 weeks of the standard multiplication protocol, the plantlets were transferred to these tuberization media. The effect of the variations in pH of the microtuberization medium was investigated in relation to number of tubers formed, their average microtuber weight, and microtuber weight distribution.

7.4 Replacing microtuberization medium

To investigate if replacing the media during the microtuberization phase had any effect, the standard tuberization medium was discarded and replaced with a fresh one of the same composition at 1, 2, 3, 4 and 5 week intervals. Microtubers formed in the different treatments were harvested 10 weeks following the start of the microtuberization phase. Harvested fresh microtubers were weighed individually.

7.5 Osmotically equivalent media

When required the media containing different monosaccharides were adjusted to be osmotically equivalent to that of 8% sucrose (80 g/l), using the van't Hoff law $\pi = m/cRT$ (Salisbury 1982). The molecular weights of carbohydrates used were 342 for sucrose, 180 for glucose and 180 for fructose. 8% sucrose solution has a molality of $80/342=0.234$. From the molecular weights, it was possible to calculate the amounts of other carbohydrates to be added compared to 8% sucrose solution: 42.1 g glucose, 42.1 g fructose and 21.05 g glucose and 21.05 g fructose mixture were added to the total volume of 1,000 ml MS basal medium to generate media that were osmotically equivalent to the standard microtuberization medium containing 8% sucrose.

In paper chromatography, the breakdown of sucrose was in autoclaved media detected. Hence, each of the monosaccharide solutions was filter-sterilized using 0.22 μM membrane filters (type GS, Millipore Corporation, U.S.A.) before being added to the rest of the autoclaved MS basal medium.

7.6 Media with initial carbon content equivalent to that of the 8% sucrose solution

In some variations to the standard microtuberization protocol, the media containing different monosaccharides were adjusted to have carbon contents equivalent to that of the standard microtuberization medium with 8% sucrose (80 g/l). The molecular weights of carbohydrates used were 342 for sucrose, which has a molecular formula of $C_{12}H_{22}O_{11}$, and 180 for glucose and fructose, which both have a molecular formula of $C_6H_{12}O_6$. 8% sucrose solution has a molality of $80/342=0.234$ and has double the amount of carbon in its structure when compared to glucose and fructose. From the molecular weights, it was possible to calculate the amounts of other carbohydrates to be added compared to 8% sucrose: $(0.234 \times 180 \times 2 =)$ 84.2 g glucose, $(0.234 \times 180 \times 2 =)$ 84.2 g fructose. A 42.1 g glucose + 42.1 g fructose mixture was added to total volume of 1,000 ml MS basal medium to generate medium that had a carbon content equal to the standard microtuberization medium containing 8% sucrose.

Each of the carbohydrate solutions was filter sterilized through a 0.22 μ M membrane filter (type GS, Millipore Corporation, U.S.A.) and added to autoclaved MS basal medium.

7.7 Medium containing maltose

8% (w/v) maltose (analytical grade, sigma, USA) solution was filtered, sterilized through 0.22 μ M membrane filters (type GS, Millipore corporation, U.S.A.) and then added to the autoclaved MS basal medium. Both sucrose and maltose have the same molecular weight (324) and formula of $C_{12}H_{22}O_{11}$.

8. Paper chromatography and HPLC analysis of sugars

Media were collected from three jars at different stages of plantlet growth and microtuber development and then filtered through Whatman No.1 filter paper.

Paper chromatography of sugars of the filtered media was carried out in ethylacetate-pyridine-water (10:4:3, v/v) and developed twice on Whatman No.1 paper (Hough and Jones 1962). Sugars were visualized with a mixture of 4 g of diphenylamine in 100ml acetone and 4ml of aniline in 96ml acetone with 20 ml of phosphoric acid as spraying

agent (Scott 1969). When media containing sucrose were autoclaved, traces of glucose and fructose could be detected by paper chromatography in aliquots of the autoclaved media. Changes of sugars in these media were also determined quantitatively by the HPLC method. Sugars were separated on a 220x4.6 mm reverse-phase amino column using acetonitrile-water (75:25, v/v) as the mobile phase (flow rate, 1.5 ml/min) at column temperature of 30 °C. The sugar peaks were measured using a refractive index detector.

9. Preparation of invertase and soluble proteins from culture media

Media from 3 jars at different stages of culture were collected and pooled together before being filtered through 1 layer of Whatman No. 1 paper.

Several difficulties were encountered including the soluble protein contents being too low for SDS-PAGE or IEF gel analyses and the frequent formation of precipitates during enzyme preparation. The following attempts were made to concentrate protein and increase the enzyme contents in solution.

- 1) The filtrates were dialyzed with 0.1 M sodium phosphate buffer or 0.1 M sodium acetate buffer at 4 °C overnight and then concentrated using polyethylene glycol (MW 20,000).
- 2) The filtrates were brought to 70% (w/v) saturation by addition of solid ammonium sulfate. The precipitates were collected by centrifugation at 15,000 r.p.m. for 15 min. (Sorvall RC-5B with rotor type SS-34, Du Pont Instrument, U.S.A.), dissolved in 1ml of 0.1 mM sodium acetate buffer (pH 5.2), and dialyzed overnight at 4°C against the same buffer. The dialyzate containing the soluble invertase activity was stored at -20 °C until enzyme assays were carried out.
- 3) Desalting column preparation of invertase solution:
Desalting process was tried to prevent precipitates from the dialyzed solution. The dialyzed solution was applied to a desalting column (3.0x5.0 cm) packed with Bio-gel HTX (Bio-rad. Ltd.), pre-equilibrated with 0.1 mM sodium acetate buffer (pH 5.2).

10. Partial purification of invertase

The dialyzed solution was applied to a DEAE-cellulose column (1.5x30 cm), pre-

equilibrated with 50 mM sodium acetate buffer (pH 5.2). The column was washed with the same buffer and then eluted with a linear gradient of 0 to 0.3 M NaCl in 500ml of the buffer. The fractions containing active invertase were pooled and designated as medium invertase.

11. Extraction of crude invertase from potato plantlet tissues

At specific times during multiplication and tuberization, roots and stems submerged in the media as well as microtubers were frozen with liquid nitrogen and 5g of each sample was ground in a mortar and pestle with 5 ml of 0.1 mM sodium acetate buffer (pH 5.2). The slurry was squeeze-filtered through three layers of moistened synthetic cloth and the supernatant fraction was retained following centrifuging at 15,000 r.p.m. for 15 min at 4 °C (Sorvall RC-5B with rotor type SS-34, Du Pont Instrument, U.S.A.). The supernatant was dialyzed in the above buffer overnight at 4 °C and the dialyzate was used as crude, cell-free invertase extracts. The enzyme extracts were stored at -20 °C.

12. Invertase assay

Invertase activity was assayed by following the enzymatic release of glucose from sucrose as substrate. Assay reaction mixture contained 200 µl of 200 mM sodium acetate buffer (pH 4.8), 200 µl of filtered culture medium, and 100 µl of 20 mM sucrose solution. Control was prepared containing 300 µl of the same buffer, 200 µl of the filtered culture medium but no sucrose solution. Mixed samples were incubated at 37 °C from 3 h to 2 days. The release of reducing sugar was determined using the HBH reagent with glucose as standard (Lever 1973). Following incubation, 20 µl of assay reaction mixture were mixed with 5 ml HBH reagent, boiled for 5 min, and then cooled to room temperature before absorbance at 490 nm was read.

For the determination of pH optima of the enzyme activities, the following buffers were used in the assay reaction mixture: 0.1 M sodium acetate buffer, pH 3.0-5.2; 0.1 M sodium phosphate buffer, pH 6.2-8.0. Each data point was the average of three replicate assays.

13. Amylase assay

Prior to the assay, media from 3 jars of cultures were collected and pooled together before being filtered through 1 layer of Whatman No. 1 paper.

The filtrates were brought to 70% (w/v) saturation by the addition of solid ammonium sulfate. The precipitates were collected by centrifugation at 15,000 r.p.m. for 15 min. (Sorvall RC-5B with rotor type SS-34, Du Pont Instrument, U.S.A.), dissolved in 2 ml of 0.1 mM sodium acetate buffer (pH 5.2), and dialyzed overnight against the same buffer. All the procedures were carried out at 4°C.

For the enzyme assay, 0.2% (w/v) soluble potato starch as a substrate was prepared fresh before enzyme assays by heating 100 mg soluble starch to boiling in 50 ml distilled water. Reaction mixture contained 0.5 ml of sodium citrate buffer (pH 5.0), 0.4 ml of the culture medium and 0.2 ml of the substrate, and was incubated at 37 °C for 30 min before 3ml of iodine reagent were added to stop the reaction. Absorbance was read at 620 nm. For enzyme control, the substrate was added following incubation of buffer and the filtered medium at 37 °C. Iodine stock for an iodine reagent was prepared by dissolving 6 g of potassium iodide and 600 mg of iodine in 100ml distilled water. Fresh iodine reagent from the stock was prepared by diluting 1 ml of iodine stock with 99 ml of 50 mM HCl.

14. Phosphatase assay

Phosphatase activity was assayed using p-nitrophenyl phosphate as a substrate. Assay reaction mixture contained 0.25 ml of 0.1 M sodium citrate buffer solution (pH 5.0), 0.25 ml of p-nitrophenyl phosphate (50 mM in the same buffer) and 0.25 ml of the medium and was incubated at 30 °C for 30 min. For control, the substrate was added after incubating the buffer and medium for 30 min at 30 °C. 0.75 ml of 1 M NaOH was added to each reaction mixture to stop the enzyme reaction and then the absorbance was read at 410 nm.

15. Protein quantification

The concentrations of soluble proteins in the filtered media were quantified using the procedure of Bradford's macroassays (1976) and bovine serum albumin (BSA) was used

as the standard.

16. SDS-PAGE

Soluble proteins were resolved by SDS-PAGE (Laemmli 1970) which was performed, in a 'purpose-built' glass-plate apparatus using 10 to 15 % acrylamide gels. Current was applied at 60mA until the dye front reached the interface between the stacking and separating gels and then the current was adjusted down to 25 mA until the tracking dye reached the bottom of the gel. After electrophoresis, polypeptides were visualized with Coomassie brilliant blue R-250 (Ougham *et al.* 1987) and the silver stain ($\text{Ag}(\text{NO}_3)_2$) method was also attempted (Chapman *et al.* 1988).

17. Non-denaturing PAGE and IEF

The procedure for non-denaturing PAGE was the same as that for SDS-PAGE except that the gels, running buffer, and the sample buffer were free of SDS or mercaptoethanol, and the invertase-containing extracts (see 11) were not heated before electrophoresis (Reisfeld *et al.* 1962). The gel concentration was investigated from 10 to 15 % acrylamide for better band resolution. Electrophoresis was carried out at 4 °C for native PAGE electrophoresis. Current was applied at 65 mA until the dye front reached the interface between the stacking and separating gels and then voltage was adjusted down to 200 V until the tracking dye reached the bottom of the gel. The gels were stained for protein with Coomassie Brilliant Blue when required.

For pI determination of invertase activity in the filtered media, thin layer isoelectric focusing in Mini-PROTEIN II (Bio-Rad Laboratories, U.S.A.) was performed according to the manufacturer's manual. Polyacrylamide gels containing ampholytes in the range of pH 3-10 were used. Voltage was applied at 200 V for 1.5 h and then the voltage was adjusted to 400 V for 1.5 h. Isoelectrofocusing was carried out at room temperature. After isoelectrofocusing, the gel was cut into pieces with reference to the band position in the lane stained for invertase activity as described in 18.3. Then the gel pieces were soaked in 50 mM KCl solution for overnight and the pH of the KCl solutions were determined.

18. Cytochemical methods for localizing invertase activity in gels

18.1 Preparation of $[\text{Ag}(\text{NH}_3)_2]^+$ solution method

Preliminary trials were attempted to localize invertase activity on the native PAGE gels using a modified cytochemical method based on $[\text{Ag}(\text{NH}_3)_2]^+$. This reagent was prepared as follows: One mol dm^{-3} of ammonium hydroxide was added to a 0.1 mol dm^{-3} solution of silver nitrate until the initial precipitate of silver hydroxide redissolved to give a clear solution. Sufficient glacial acetic acid (0.1 mol dm^{-3}) was added dropwise to change the pH either to 5.0 or to 7.0. This solution was added to sucrose (analysis grade) to give a final concentration of $0.025 \text{ mol dm}^{-3}$ and 1% agar.

The solution was autoclaved and cooled to 45°C , poured into sterile Petri dishes and allowed to solidify. The preparations were kept in the dark at 4°C until needed. A layer of the silver agar preparation was placed over a native PAGE gel on which the enzyme was separated and incubated for 2 h at 37°C .

18.2 Tetrazolium salt reaction for detection of invertase activity following gel electrophoresis

Another attempt to localize invertase on the native PAGE gels involved preparing a tetrazolium staining solution. The staining solution was prepared as follows; 85 mM citrate/phosphate buffer adjusted to pH 6.0 containing 100mM sucrose, 0.33mg/ml phenazine methosulfate and 0.02 mg/ml glucose oxidase. This staining solution was prepared and used in complete darkness. After native electrophoresis the gel plate was immersed in this staining solution and incubated at 37°C overnight. To stop enzymatic staining the gel plate was immersed for 5 min in 10% (w/v) trichloroacetic acid.

18.3 The use of glucose oxidase, peroxidase and 3,3'-diaminobenzidine (D.A.B.) for detection of invertase activity following gel electrophoresis

Invertase activity was visualized after native gel electrophoresis or thin layer isoelectric focusing gel electrophoresis as described by Faye (1981). For staining the gels, the following reaction mixture was used: 85 mM citrate/phosphate buffer adjusted to pH 6.5

containing 100 mM of sucrose, 0.02 mg/ml glucose oxidase (Sigma Chemical Co., U.S.A.), 0.12 mg/ml horseradish peroxidase (Sigma Chemical Co., U.S.A.), and 0.3 mg/ml 3,3-diaminobenzidine tetrahydrochloride (BDH Laboratory Supplies Poole, England). As a control one of the enzyme preparations was boiled and loaded to a sample well before the electrophoresis process. The gels were rapidly washed after electrophoresis with 85 mM citrate/phosphate buffer before incubation in the soluble staining reaction mixture. Then the gels were incubated at 37 °C in a moist chamber overnight. To stop enzymatic staining and to increase contrast the gels were immersed in 10% (w/v) trichloroacetic acid solution for 5 min.

19. Ultrastructural analysis

The external morphological modifications of green and white roots, stems, tubers, leaves and floating cells collected after the multiplication step and microtuberization step were examined. The microplants formed in light and microtubers formed in dark were dissected and prepared for transmission electron microscopy. Tissues were fixed in 3.0% glutaraldehyde (biological grade) in 0.025 M phosphate buffer (pH 7.0) for 4 h or overnight at 20 °C, washed in the same buffer and then post-fixed in 2% osmium tetroxide in the same buffer for 12 h or overnight at 4 °C. Dehydration in acetone at 10% stages for 10 min each up to the 90% stage, followed by three changes of 100% acetone over 1 hour and embedding in Epon araldite epoxy resins, were carried out by standard methods. Ultrathin sections were cut on a LKB ultramicrotome using a diamond knife, stained with saturated uranyl acetate for 5 min and observed using a Hitachi HS-7S electron microscope.

20. Osmotic Potential Measurements

The osmotic potentials of culture media, after filtration as described previously, were measured and readings obtained directly from a Vapor Pressure Osmometer (5500 Wescor, Utah, U.S.A., standard deviation < 2 mmol/kg). 1 µl of medium was applied to a sample disc (SS-033, Wescor, Utah, U.S.A), 6 mm diameter, following the manufacturer's

instructions for the Osmometer.

21. Mineral Analysis

The culture media, after filtration as described previously in 8, were sent to the Institute of Environmental Science and Research Limited (Wellington Science Center, New Zealand) for analysis of mineral contents via ICP mass spectrometry. The stated units used are g/m³ (or mg/l).

22. Data Analysis

The data on microtuber number and average microtuber weight were evaluated using the Analysis of Variance (ANOVA) of Statistix for Windows at a significance level $p \leq 0.05$ unless stated otherwise. Microtuber fresh weight distributions were classified according to 5 or 6 categories (≤ 0.25 , 0.25-0.50, 0.50-0.75, 0.75-1.0, >1.0 g and >2.0 g) and converted to percentages for comparison.

III. RESULTS

A. Preliminary trials with different potato microtuberization systems

In liquid medium, the plantlets that were completely submerged could not form tubers when incubated in darkness for 10 weeks. The depth of the liquid medium was also important during the multiplication stage. If the liquid level was too low, the growth of plantlets was inhibited. When the liquid level was too high, further growth of stems, roots and leaves became retarded and the stem became stoloniferous. Depth control of the medium is therefore an important consideration in the use of liquid media for potato microtuberization.

Of the 4 microtuberization systems trialled here (Fig.1), the stationary system appeared to be most promising as far as microtuberization is concerned (Table 1). This was adopted for further investigation in this thesis.

A preliminary examination of the possible involvement of invertase activity in the medium for sucrose which supplied during microtuberization was also carried out. It was found that invertase activity was much higher in the 8% sucrose medium than that in the media containing other carbohydrates (Table 2). It is also interesting that amylase activity was not detected in any of these media while acid phosphatase activity was similar in all of the media. Taken together, the relatively high invertase activity seemed to be associated with 8% sucrose tuberization medium.

The standard two-step stationary culture procedure used throughout the thesis was modified slightly from the preliminary stationary culture system. In the first step of the preliminary system, three plantlets with 2 nodes each were floated in 25ml of liquid propagation medium containing 3% sucrose in MS basal salt solution (depth about 10mm high). This first stage is to promote vegetative growth prior to microtuberization. It was observed that vegetative growth was important to get a bigger microtuber in the next tuberization step. Normally, roots and lower parts of the plantlets were submerged, but upper parts (stem and leaf) of the plantlets remained over the surface of the aerial space in

liquid medium within a 250ml tissue culture container (64mm ID x 80mm).

After 4 weeks, when the plantlets were well established, the residual medium was drained off and replaced to increase medium depth (15-20 mm) with 50ml of new microtuberization medium containing 8% sucrose in MS basal medium.

In the second stage, the plantlets were incubated at 20 °C in darkness. After 10 weeks, the microtubers were harvested.

CULTURE MANIPULATION

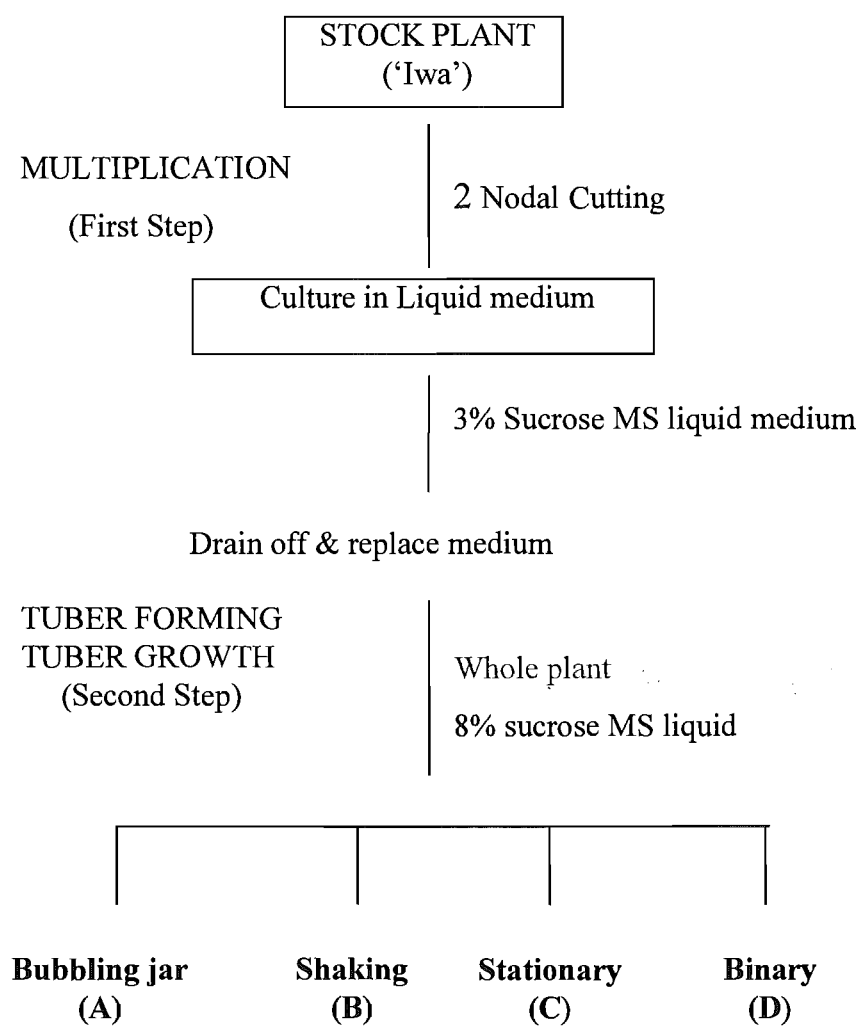


Fig. 1. Preliminary trials of 4 different microtuberization procedures.

Please note: throughout the thesis, the *in vitro* plantlets of the 2 potato cultivars used for experimentation are denoted as ('Iwa') and ('Daeji').

Table 1. Results of the preliminary trials in Fig. 1 at 10 weeks from the start of the microtuberization step.

	A	B	C	D
Earliness of induction	+++	+	+++	++
Microtuber numbers	++	+	+++	++
Tuber size	+++	+	+++	+++
Tuber yield	+++	+	+++	+
Appearances	Thick stems	Most tissues submerged in the medium; vitrification evident	Thick stems	Poor tuber growth rate; low productivity

Key: +++ good or most advanced
++ intermediate level
+ poor or less advanced

Table 2. Presence of enzyme activities in the medium at the end of the preliminary tuberization experiments with ‘Iwa’ plantlets.

Medium ^a	Enzyme Activity ^b		
	Invertase (nM/h/mg/protein)	Phosphatase (nM/h/mg/protein)	Amylase (nM/h/mg/protein)
8% sucrose	300	1600	0
Glucose	53	1200	0
Fructose	94	1400	0
Glucose+Fructose	14	1200	0
Maltose	00	1700	0

- a. MS basal medium + the appropriate carbohydrates. All media except the 3% sucrose medium were osmotically equivalent to the 8% (w/v) sucrose medium at the beginning of the *in vitro* tuberization step.
- b. Presence of the indicated enzyme activities in the media after 10 weeks from the start of the *in vitro* tuberization step was examined. The activity of the enzymes assayed was expressed as the nM reaction product formed per hour per mg of protein indicated.

B. Main Experiments

1. Effect of manipulations of tuberization medium on microtuberization

1.1 Standard Protocol

1.1.1 Time course of microtuber development

In vitro potato plantlets (cvs. 'Iwa' and 'Daeji') with an upright vigorous shoot and a well-developed, long green root, cultured in a stationary liquid tuberization medium with 8% sucrose for 10 weeks in darkness, formed about 3.0 microtubers per 3 plantlets per jar. All the microtubers showed marked increase in fresh weight within 7 weeks of culture; after from then until 10 weeks there appeared to be little or no further change (Fig. 2).

1.1.2 Carbohydrate changes in the standard tuberization medium

Composition of sugars in the medium exhibited remarkable changes while the microtubers were formed (Figs. 3a and 3b). After 2 weeks in culture, the medium contained large amount of glucose and fructose in addition to sucrose (Figs. 4a and 4b). The decrease of sucrose in the medium was correlated with an increase in the levels of glucose and fructose. After 6 weeks in culture, sucrose was almost completely converted (i.e. not detectable with paper chromatography but trace amount found in HPLC analysis) to glucose and fructose, which were present in similar proportions. By week 8, sucrose was no longer detectable even in HPLC analysis, but the levels of glucose and fructose in the medium peaked. Then these sugars decreased in the medium slightly and in similar amounts although this seems to have happened faster in the case of 'Daeji' than in 'Iwa' (Figs. 4a and 4b).

It was apparent that the decrease in sucrose concentration of the medium accompanied the beginning of microtuber formation and correlated notably with the cessation of microtuber development.

1.1.3 Invertase activity in the standard tuberization medium

1.1.3.1 Preparation of invertase from the medium

Since invertase level appeared to be low in large volume of the liquid medium, it was necessary to concentrate the enzyme. To this end, several general protein

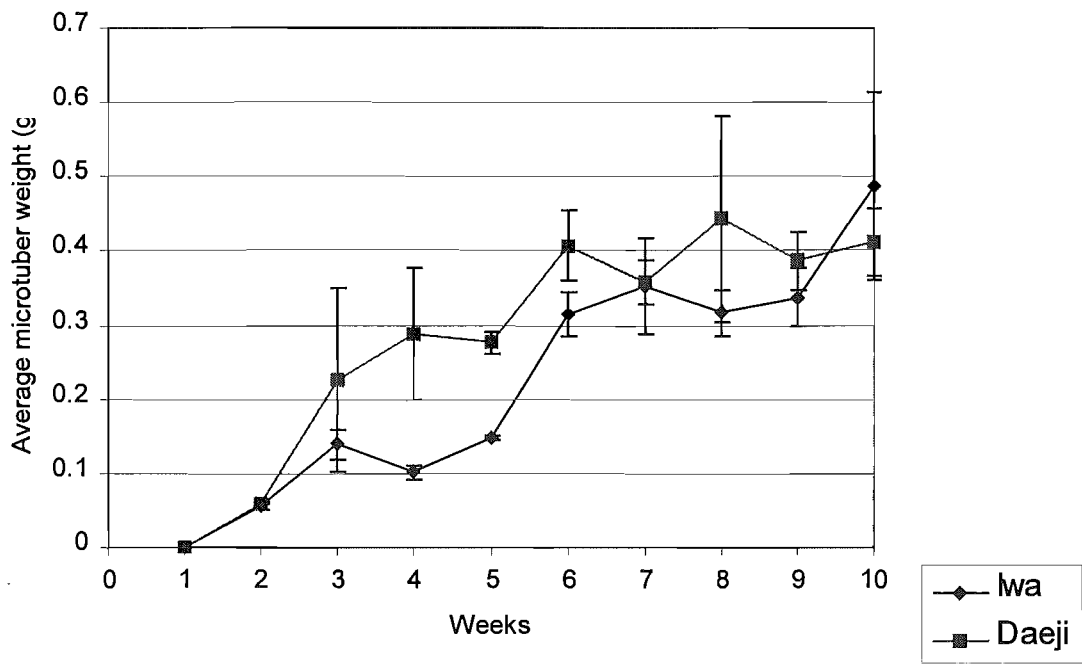


Fig. 2. Time course of microtuber growth ('Iwa' and 'Daeji'). The microtubers harvested at the end of the microtuberization step (10 weeks) from each medium type with a certain sucrose concentration was individually weighed shortly after harvest. Each value represents the average tuber weight from two replicates jars.

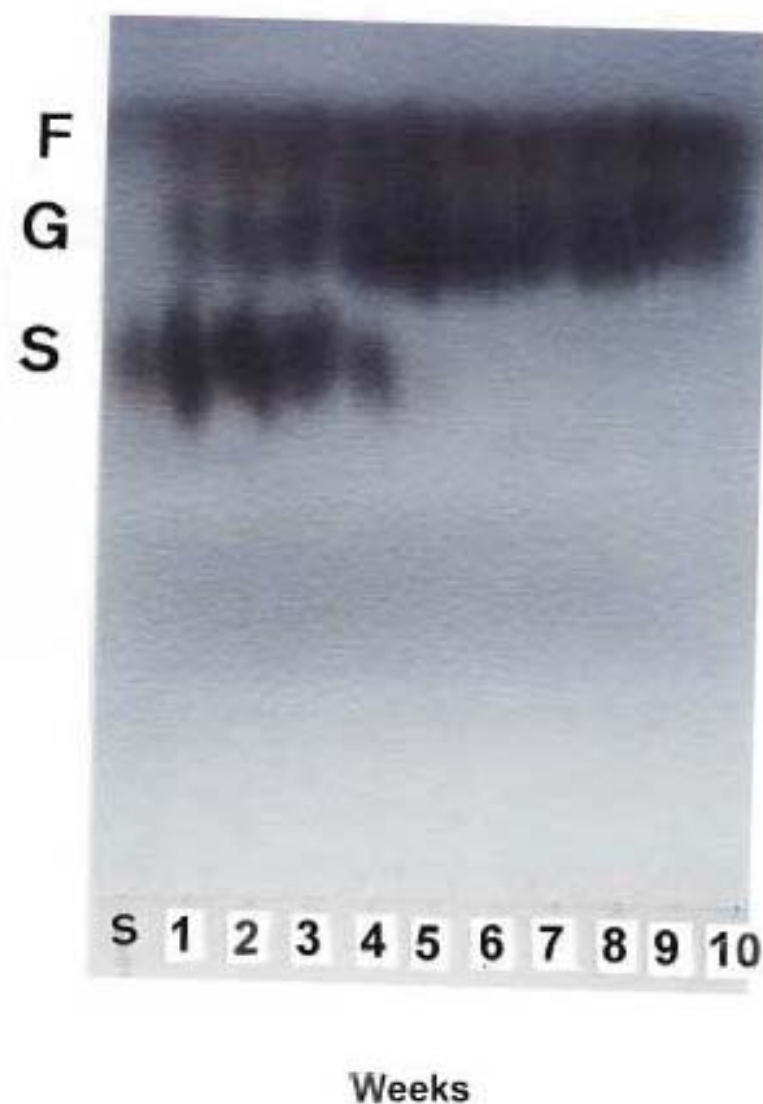


Fig. 3a. The paper chromatogram of time course of carbohydrate changes in the standard tuberization medium ('Iwa'). Sugar standards F=fructose, G= glucose and S= sucrose.



Fig. 3b. Time course of carbohydrate changes on paper chromatography in the standard tuberization medium ('Daeji'). F= fructose, G= glucose and S= sucrose.

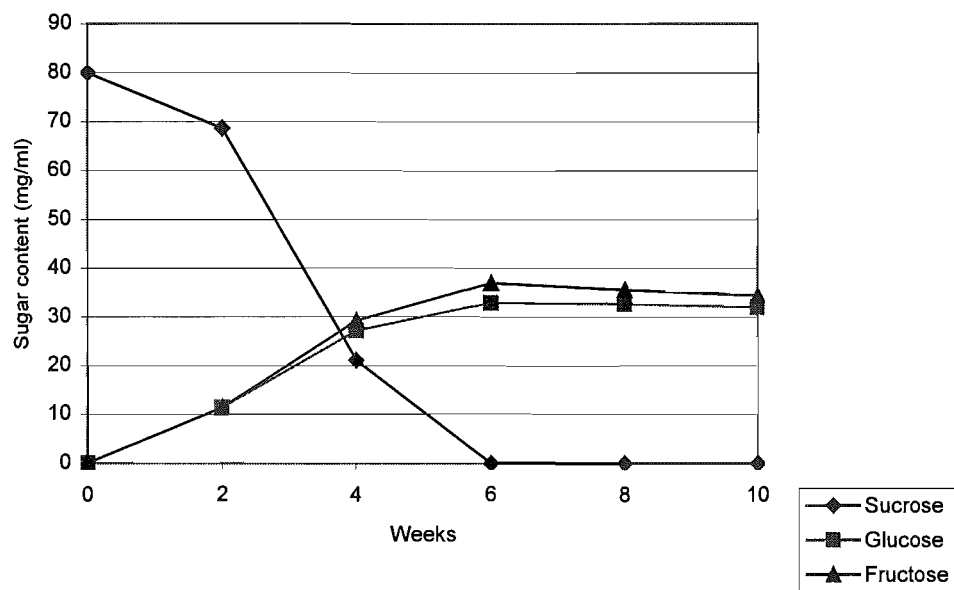


Fig. 4a. HPLC analysis of carbohydrate changes in the standard tuberization medium ('Iwa').

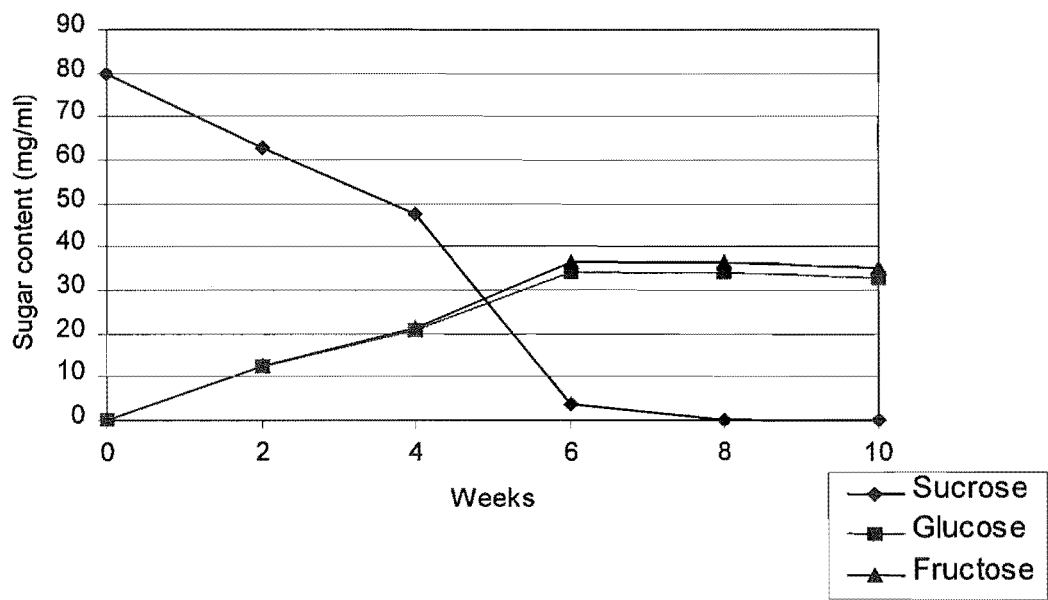


Fig. 4b. HPLC analysis of carbohydrate changes in standard tuberization medium ('Daeji').

concentration methods were attempted, including PEG sorption and ammonium sulphate precipitation. A further challenging problem was encountered: proteins from the medium tended to form precipitates during dialysis in buffer solution, or upon storage at 4 °C. Freshly prepared samples were used to avoid these problems.

1.1.3.2 Optimum pH of invertase activity

The enzyme activity in the medium for microtuberization of 'Iwa' was determined over a broad pH range (Fig. 5). The peak activity at around pH 5.0 suggests that the sucrose hydrolyzing activity in the medium was an acid invertase and not an alkaline invertase.

1.1.3.3 Change of invertase activity in the medium during tuberization

The development of invertase activity in the medium for microtuberization of 'Iwa' and 'Daeji' correlated well with the initiation and development of microtubers (1.1.1) and with carbohydrate changes in the medium (1.1.2). The enzyme activity increased in the first 6 weeks and then started to fall in the next 4 weeks (Fig. 6). The peak level of invertase activity in the medium for tuberization of 'Daeji' plantlets was higher than that for 'Iwa' and the subsequent decline in the case of 'Daeji' was also more substantial than in that of 'Iwa'.

1.1.3.4 Presence of invertase in parts of stems and roots that were submerged in liquid medium

Submerged stems and roots of 'Iwa' plantlets contained invertase activities but the presence of invertase in microtubers was not detected. The dependence of enzyme activities in roots or stems on pH ranging from 2 to 8 is shown in Fig. 7. The extract of submerged potato stems and roots had two peak activity at around pH 4 and 6. There was no evidence for alkaline invertase activity in either extract.

1.1.3.5 Invertase Isozymes

To determine the number of invertase isoenzymes in the tuberization medium, non-denaturing

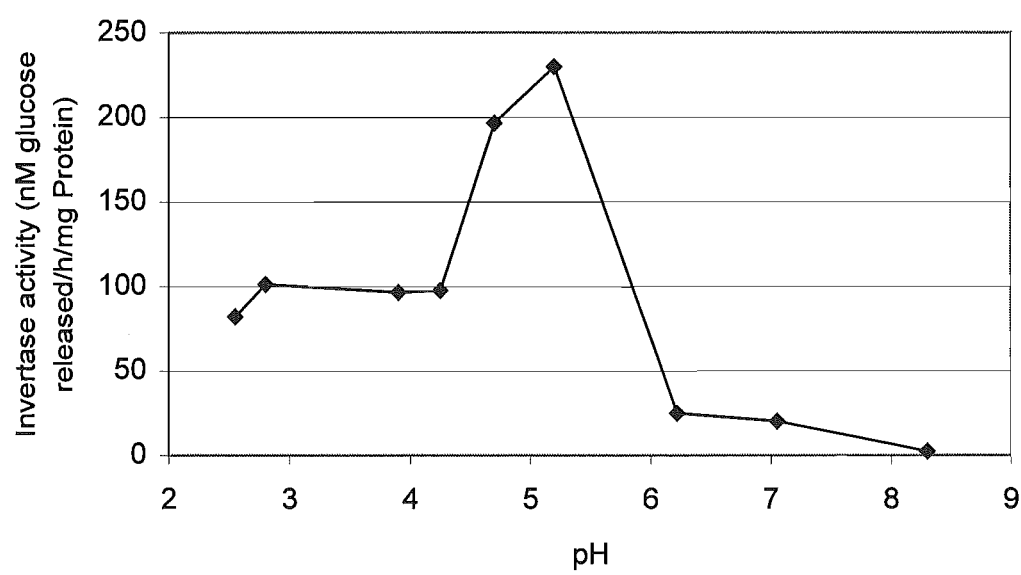


Fig. 5. pH profile of invertase activity in the standard microtuberization medium ('Iwa').

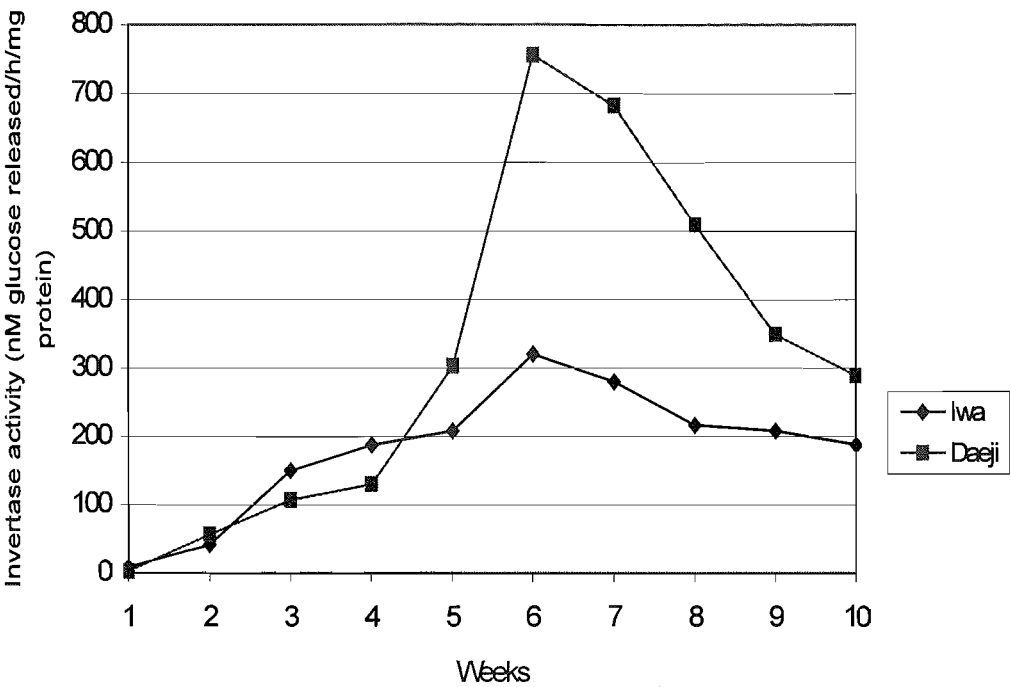


Fig. 6. Time course of invertase activity development during microtuberization in standard tuberization medium ('Iwa' and 'Daeji').

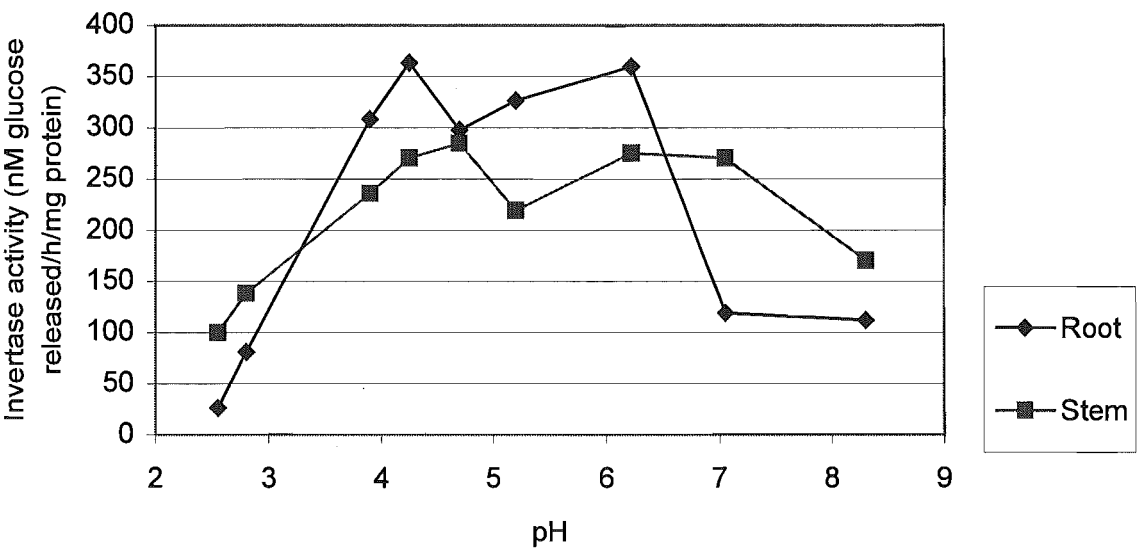


Fig. 7. pH profiles of invertase activities in the extracts of the plantlets 'Iwa'.

PAGE and non-denaturing thin layer isoelectric focusing gel electrophoresis were carried out and then several methods were attempted to detect the enzyme activity on the gels. The most satisfactory method involved the use of an auxilliary chromogenic system (Faye 1981). The gels immersed in the soluble staining system overnight showed a single stained band in each sample lane on the gel (Fig. 8) with pI at around 6.75. The same band appeared to be present in root extract and in multiplication medium.

1.1.3.6 Changes in soluble protein content during tuberization

Soluble protein content of the medium changed during tuberization of 'Iwa' plantlets (Fig. 9). There was a sharp increase in 4-5 weeks reaching a maximum at/after 6 weeks in culture. Subsequently there was little or no further change in the next 4 weeks.

1.1.4 SDS-PAGE of proteins in the standard tuberization medium

Analysis by SDS-PAGE of crude protein preparations from the standard microtuberization medium revealed a number of proteins bands ('Iwa'). One of the major bands had an apparent molecular mass of 30 kD (Fig. 10).

1.1.5 pH changes in the medium during *in vitro* tuberization

The initial pH of the tuberization medium changed in the course of sucrose cleavage in the medium during microtuber initiation and development ('Iwa'). There was a decline of the pH to 4.3 during the first 2 weeks of culture. Then the pH started to increase to around 5 for the rest of the experiment (Fig. 11). This coincided with the completion of sucrose cleavage in the medium.

1.1.6 Osmotic potential change in the standard tuberization medium

Osmotic potential of the medium was also measured to determine the correlation between osmotic potential changes and sucrose cleavage in the medium (Fig. 12). It rose between 2 and 4 weeks reaching a peak at week 4 during *in vitro* tuberization of 'Iwa' plantlets, and

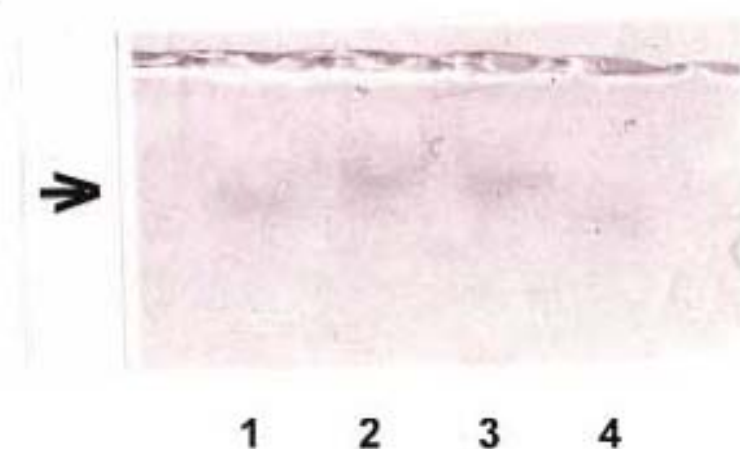


Fig. 8. Isozyme gel analysis of invertase showing a single band in each lane on the gel with pI at around 6.75. Lane 1: stem, Lane 2: root, Lane 3: microtuberization medium, Lane 4: multiplication medium.

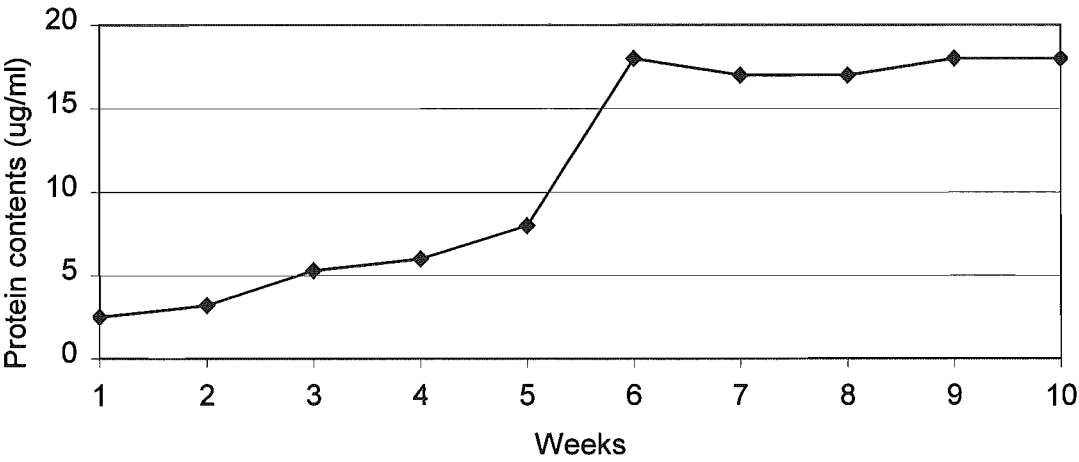


Fig. 9. Time course of changes in the soluble protein content in the microtuberization medium ('Iwa').

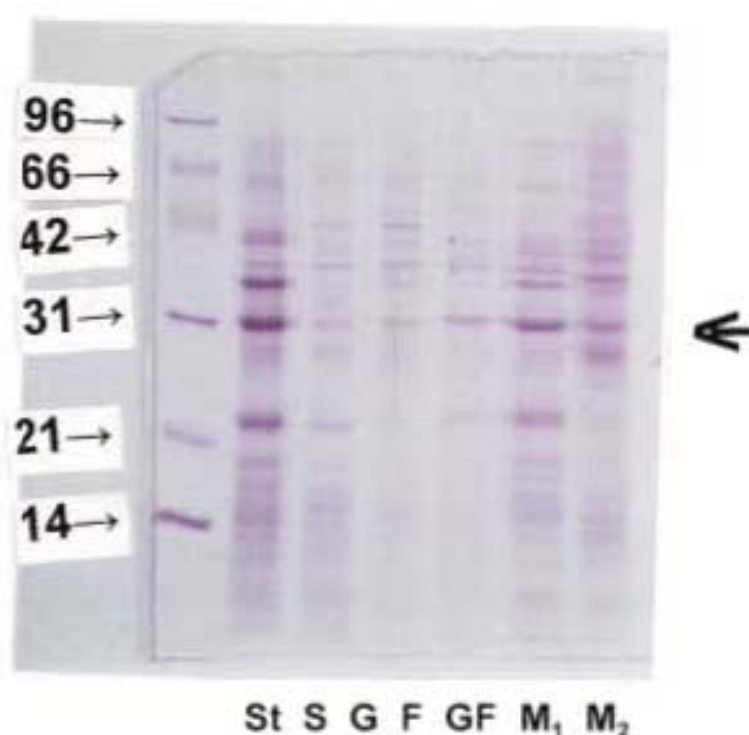


Fig. 10. Analysis by 12.5% SDS-PAGE of samples from partially purified liquid media. A major band with apparent molecular mass of 30 kD (indicated with arrow head) was noted in all samples.

Lane St= molecular marker,

Lane S= standard microtuberization medium containing 8% sucrose,

Lane G= standard microtuberization medium containing 8% glucose,

Lane F= standard microtuberization medium containing 8% fructose,

GF= standard microtuberization medium containing 4% glucose + 4% fructose,

M₁= standard microtuberization medium containing 8% maltose,

M₂= standard multiplication medium containing 3% sucrose.

All the microtuberization media were harvested after 10 weeks of culture and the plantlet multiplication medium was harvested after 4 weeks of culture.

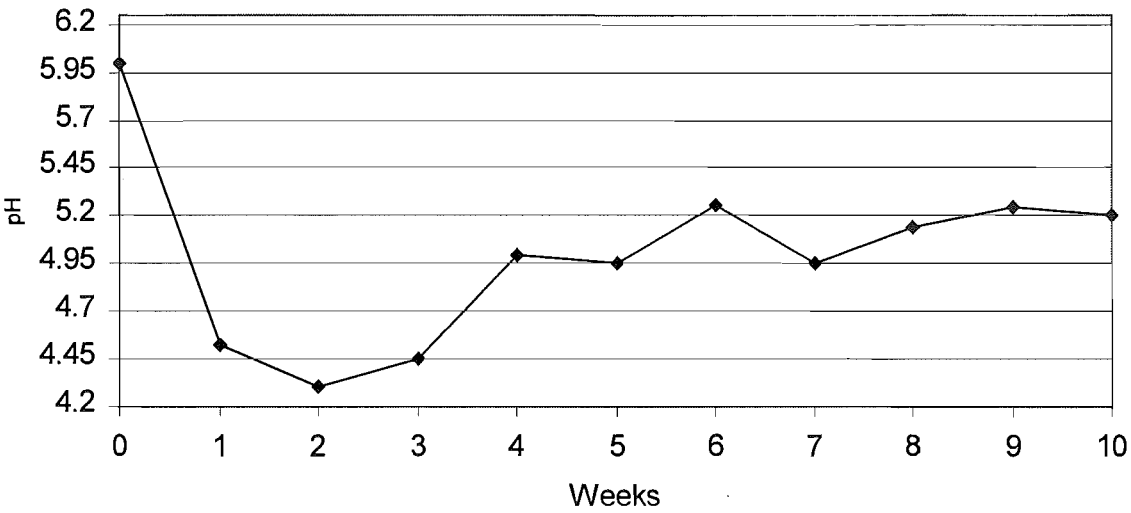


Fig. 11. Time course of pH changes in the medium during *in vitro* tuberization ('Iwa').

then dropped slightly but remained at a high level up to 10 weeks. A similar pattern of changes in the osmotic potential of the medium was observed during tuberization of 'Daeji', except that it peaked at 3 weeks and at a smaller magnitude.

1.1.7 Time course of mineral changes in the standard tuberization medium

The levels of several major inorganic ions in the medium were measured in 'Iwa' plantlets to identify whether mineral nutrients could contribute to the osmotic potential changes in liquid medium. Among the minerals studied potassium and calcium contents decreased more rapidly within the first 2 weeks of *in vitro* tuberization of 'Iwa' plantlets than later on (Fig. 13).

1.2 Effects of varying the concentrations of sucrose in the tuberization medium

1.2.1 Effect on microtuber formation

Following 4 weeks of the standard plantlet multiplication protocol, the effects of culturing the plantlets in media containing varying amounts of sucrose ranging from 0 to 20% (w/v) on the number of microtubers formed, their average fresh weights and relative size distribution were investigated.

Microtubers were initiated in the plantlets of both varieties 'Iwa' and 'Daeji' 2 weeks after transfer from the multiplication medium to a wide range of sucrose-containing media, except the sucrose-free medium on which the plantlets did not form microtubers after 10 weeks in darkness. The number of microtubers formed by 'Iwa' varied from 2.0 to 3.3 per 3 plantlets in media containing sucrose concentrations ranging from 2 to 20%, with 4 to 8% appearing to be the best treatments (Table 3). Similarly, the number of microtubers formed by 'Daeji' plantlets did not seem to vary much among the different sucrose treatments, possibly with the exception of the 20% sucrose medium (Table 3).

Overall the 'Iwa' plantlets appeared to form heavier microtubers than those of 'Daeji' in response to sucrose ranging from 2% to 20% in the media (Figs. 14 and 15).

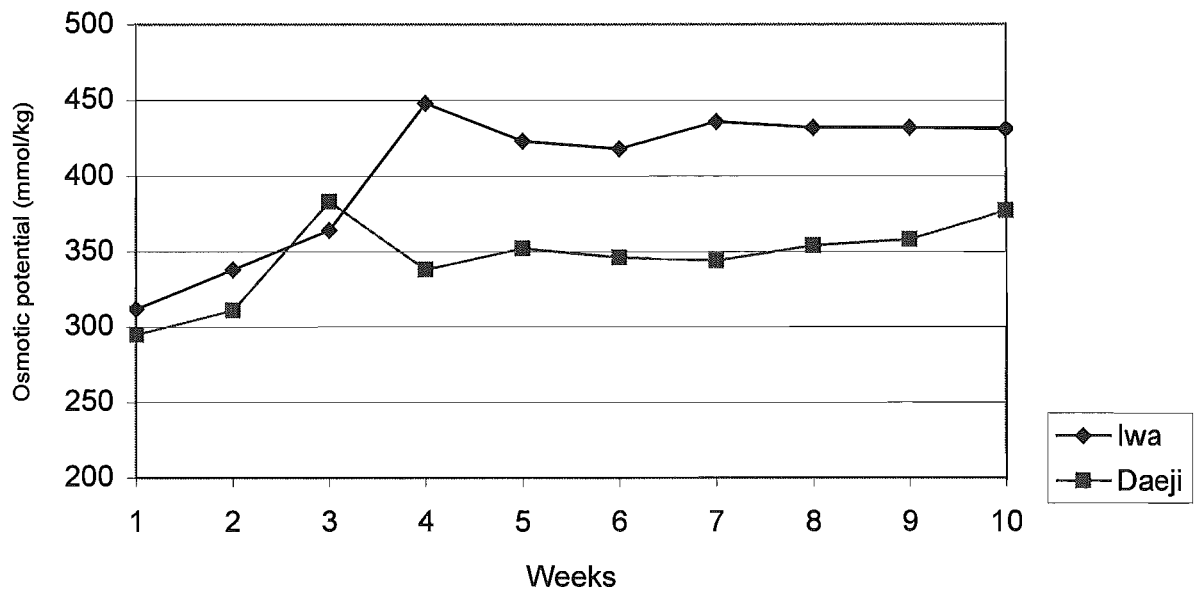


Fig. 12. Time course of osmotic potential changes in the standard microtuberization medium ('Iwa' and 'Daeji').

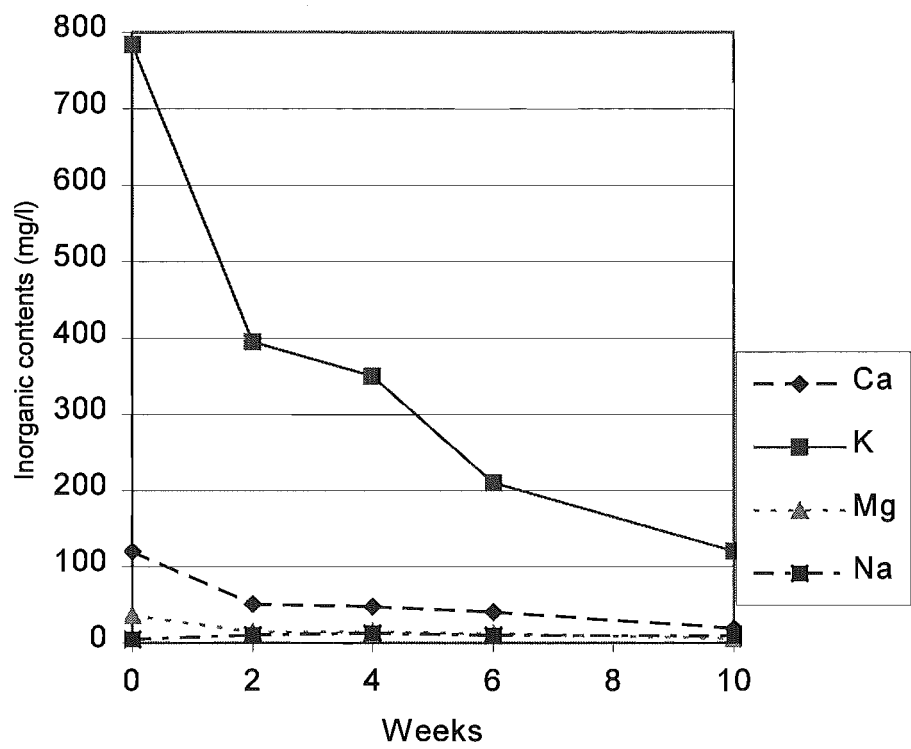


Fig. 13. Time course of major inorganic ion changes in the standard microtuberization medium ('Iwa').

Table 3. Effect of varying sucrose concentration on number of microtubers formed after 10 weeks in the dark.

Number of microtubers produced per 3 plantlets in a jar

Sucrose concentration in media (% w/v)	'Iwa'	'Daeji'
0	0	0
2	2.33 ± 1.06^{ab}	3.83 ± 0.56^a
4	3.16 ± 0.56^b	3.20 ± 0.70^a
6	3.25 ± 0.25^b	3.00 ± 1.00^a
8	2.66 ± 1.06^b	2.57 ± 0.61^{ab}
12	3.00 ± 1.11^b	3.25 ± 0.50^a
16	2.66 ± 1.06^b	3.50 ± 1.90^a
20	2.00 ± 0.12^a	1.66 ± 0.16^b

Superscripts with same letter are not significantly different according to Bonferoni comparison of means at the 0.05 level.

Microtuber size in the case of 'Iwa' increased from 0.43g (average weight from three plantlets) in 2% sucrose medium to 0.60g in 6% sucrose medium and decreased from 0.79g in 8% sucrose medium to 0.32g in 20% sucrose. Similar trends were observed with 'Daeji' (Fig. 15).

An examination of the frequencies of microtubers in different weight categories (Figs. 16 and 17), confirms that 8% sucrose, the concentration used in the standard tuberization medium, was the best tuberization medium and tended to favour the formation of larger microtubers and fewer smaller ones in both varieties. The 2%, 16% and 20% sucrose media resulted predominantly in the production of small microtubers (mainly less than 250mg).

1.2.2 Effect on soluble protein contents of the tuberization media

Changes in soluble protein contents ('Iwa') could be detected in the medium during tuberization. The soluble protein contents of the tuberization media supplemented with varying concentration of sucrose increased sharply reaching a maximum after 6 weeks in culture (Fig. 18). In general, higher sucrose media tended to have higher contents of soluble proteins. Subsequently there was little or no further change in the next 4 weeks.

1.3 Effects of different carbohydrates in the tuberization medium on microtuberization

1.3.1 The initial osmolality of the media containing different monosaccharides was equivalent to that of 8% sucrose (i.e. the standard tuberization medium)

1.3.1.1 Microtuber formation

Following 4 weeks of the standard plantlet multiplication protocol, the effects of culturing the plantlets in tuberization media supplemented with glucose and fructose, either singly or in combination, were studied in comparison with standard 8% sucrose medium. The initial osmolality of all these media was adjusted to be the same. The number of microtubers formed, their average fresh weights and relative size distribution were investigated. Microtubers were initiated in the plantlets of both varieties 'Iwa' and 'Daeji' 2 weeks after transfer from the multiplication medium to the different tuberization media.

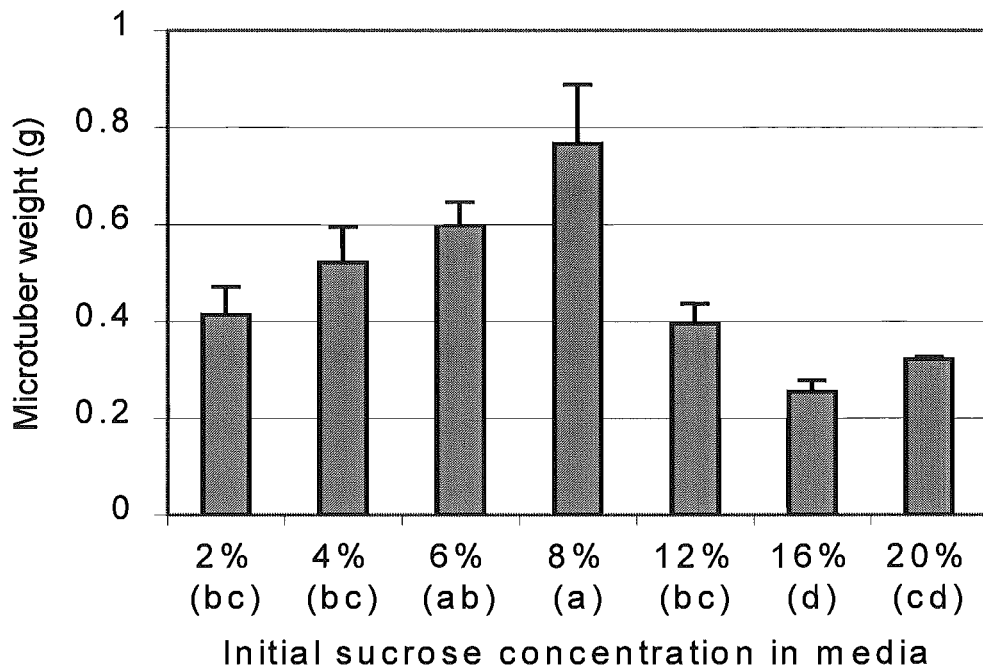


Fig. 14. Effect of sucrose concentrations on average fresh microtuber weight ('Iwa'). The microtubers harvested at the end of the microtuberization step (10 weeks) from each medium type with a certain sucrose concentration were individually weighed shortly after harvest. An average of the fresh weights of individual microtubers was computed. Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.05 level.

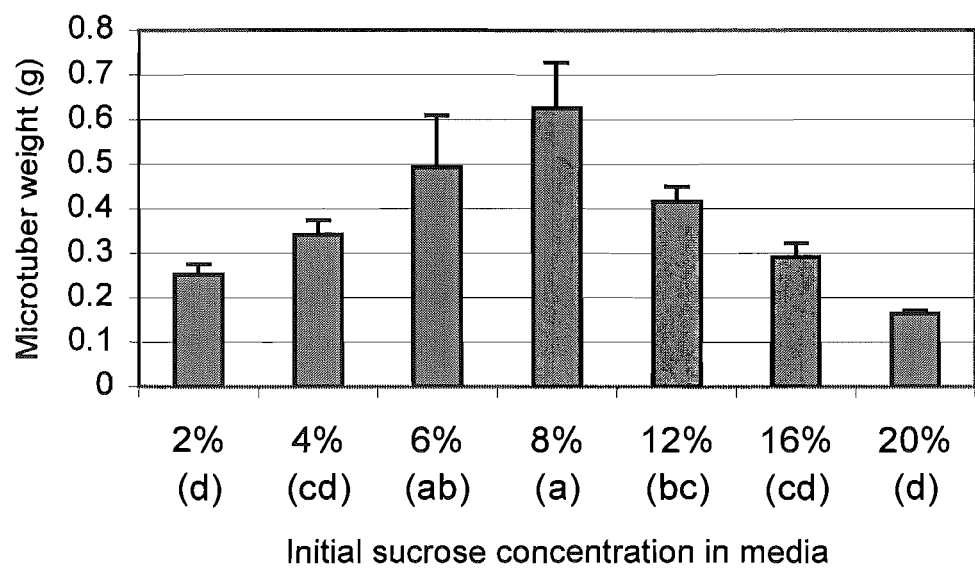


Fig. 15. Effect of sucrose concentrations on average fresh microtuber weight ('Daeji'). The microtubers harvested at the end of the microtuberization step (10 weeks) from each medium type with a certain sucrose concentration were individually weighed shortly after harvest. An average of the fresh weights of individual microtubers was computed. Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.05 level.

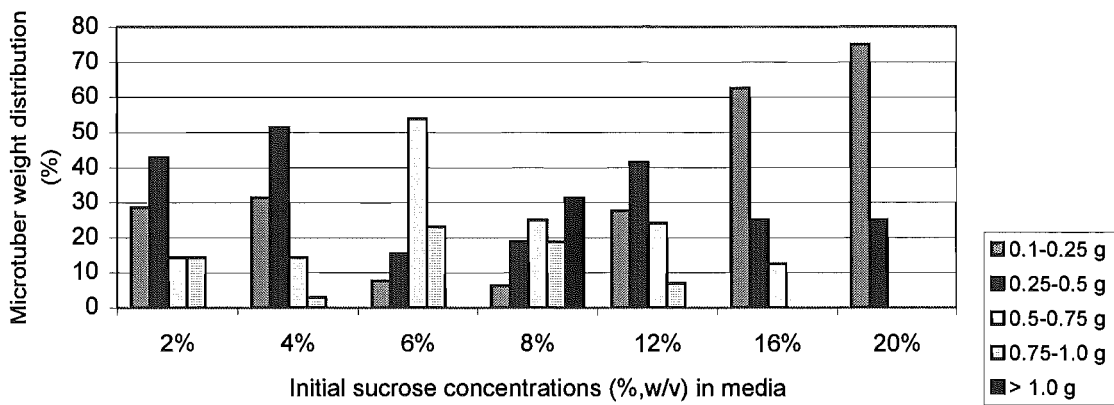


Fig. 16. Microtuber fresh weight distribution in response to different sucrose concentrations in the medium ('Iwa').

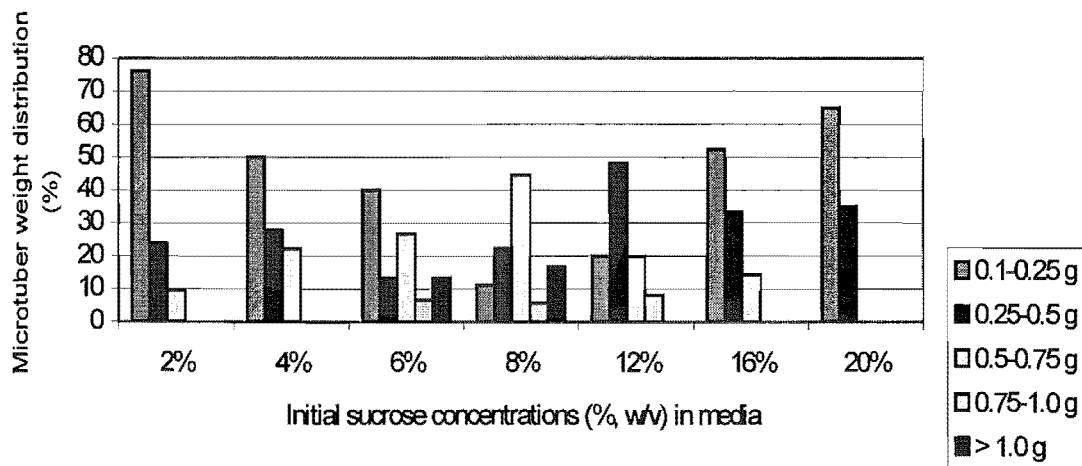


Fig. 17. Microtuber fresh weight distribution in response to different sucrose concentrations in the medium ('Daeji').

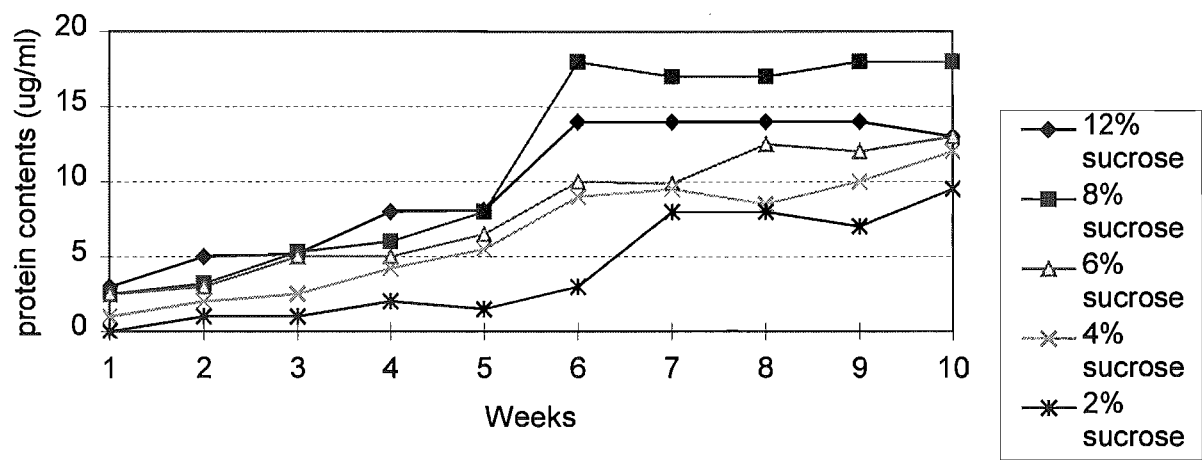


Fig. 18. Soluble protein changes in the media containing different sucrose concentrations during tuberization ('Iwa').

The number of microtubers formed in a jar each containing 3 plantlets was similar in the glucose and sucrose media, whereas it seemed higher in the fructose medium and that with a mixture of glucose and fructose (Fig. 19) although the statistical analyses revealed no significant differences among the 4 treatments ($p \leq 0.05$). In contrast, the response of 'Daeji' plantlets to these media was different. The fructose or sucrose medium appeared to have similar a effect and a number of microtubers formed was apparently higher ($p \leq 0.05$) than in the glucose or glucose + fructose medium (Fig. 20).

It seems that 'Iwa' plantlets produced heavier microtubers in the medium initially supplied with a mixture of glucose and fructose or the standard 8% sucrose microtuberization medium than when glucose or fructose was supplied singly to the tuberization medium (Fig. 21). The results obtained with 'Daeji' plantlets (Fig. 22) were different and showed that the response to the different media were very similar overall although ANOVA might show otherwise.

An examination of the frequencies of microtubers in different weight categories formed by 'Iwa' plantlets showed clearly that the tuberization medium containing sucrose or a mixture of glucose and fructose rather than medium containing glucose or fructose supplied singly tended to favour the production of larger microtubers and fewer smaller ones, particularly those less than 250mg (Fig. 23). A similar frequency of microtubers in different weight categories in response to the different media were also observed with 'Daeji' plantlets (Fig. 24). In particular, the medium with glucose supplied singly resulted predominantly in the production of smaller microtubers.

1.3.1.2 Osmotic potential changes of the different monosaccharide-containing media during microtuberization

The initial osmotic potential of the different monosaccharide media was adjusted to be the same as that of the 8% sucrose medium (Fig. 25 and 26). During microtuberization of 'Iwa' plantlets, the osmotic potential of the sucrose medium, which was included for comparison, first dipped slightly before increasing sharply within the first 4 weeks (Fig. 25). By contrast, that of the other 3 media decreased. Then while the osmotic potential of the sucrose medium decreased slightly before stabilizing at a relatively high level till the end of the

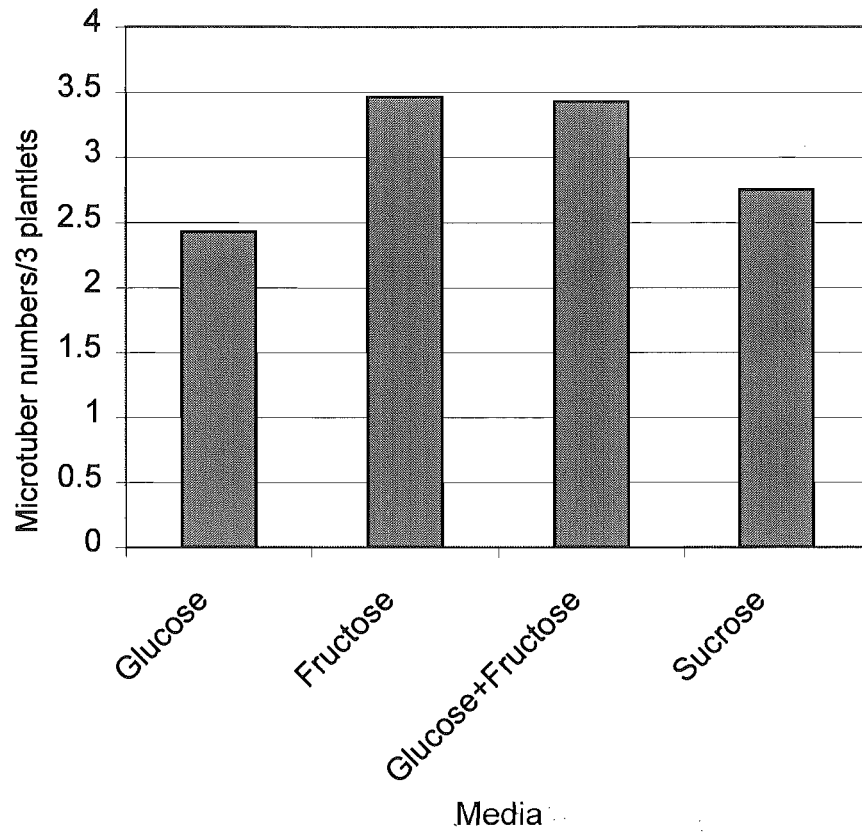


Fig. 19. Effect of media osmotically equivalent to the 8% sucrose medium at the onset of the *in vitro* tuberization step on the number of microtubers formed ('Iwa'). No significant difference was found among the treatments (ANOVA, $p \leq 0.05$)

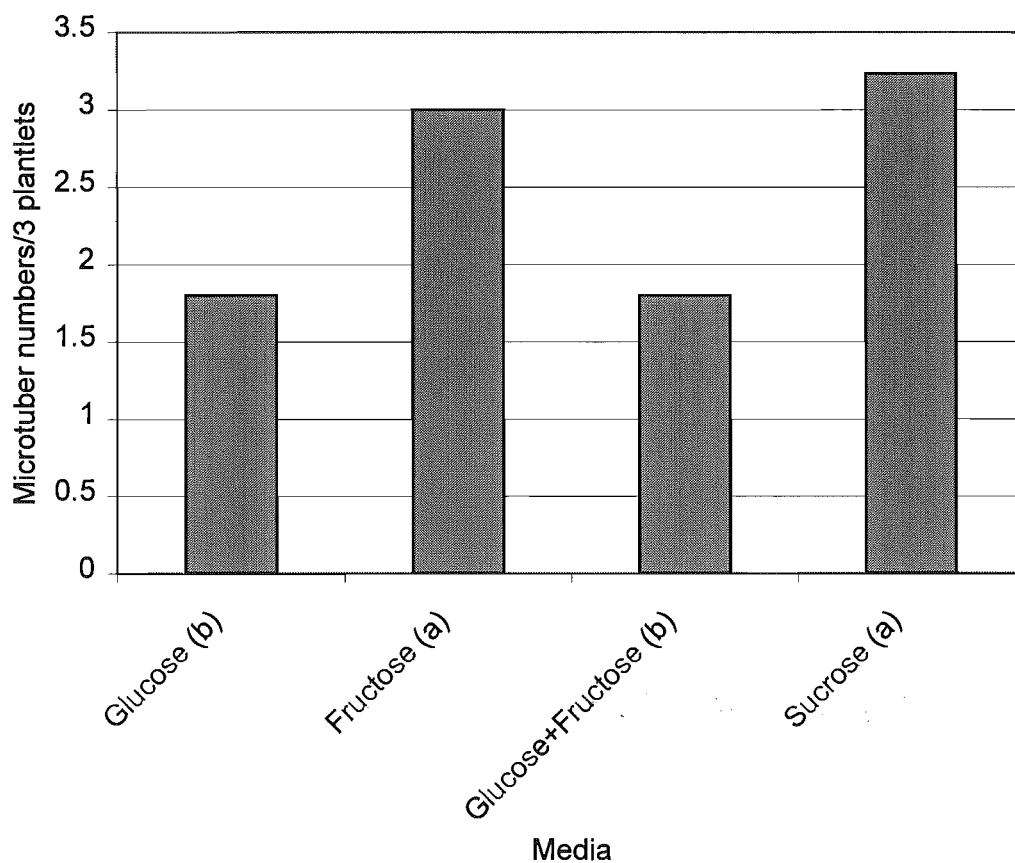


Fig. 20. Effect of media osmotically equivalent to the 8% sucrose medium at the onset of the *in vitro* tuberization on number of microtubers formed ('Daeji'). Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.001 level.

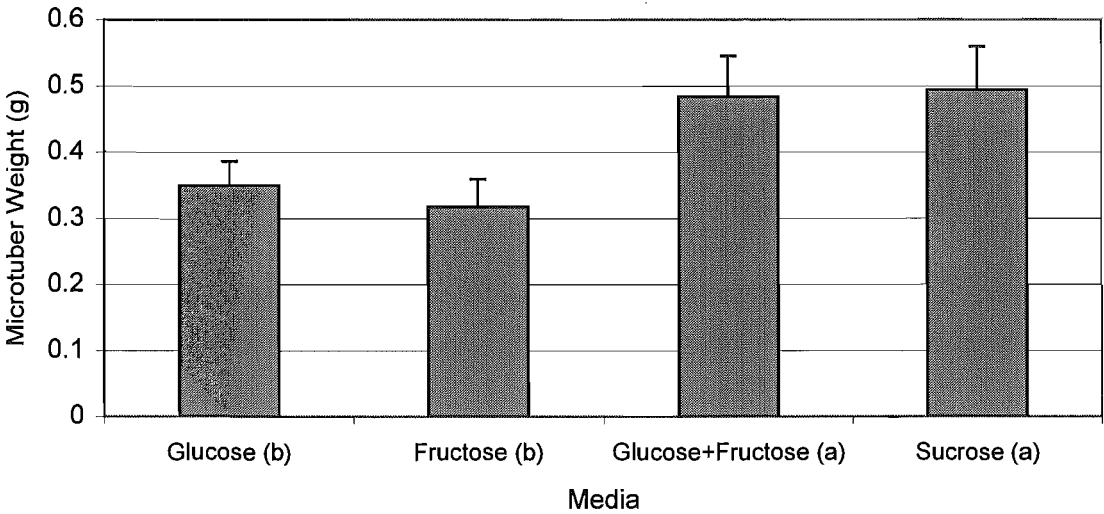


Fig. 21. Average microtuber fresh weight in response to media osmotically equivalent to the 8% sucrose medium at the beginning of microtuberization step ('Iwa'). Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.01 level.

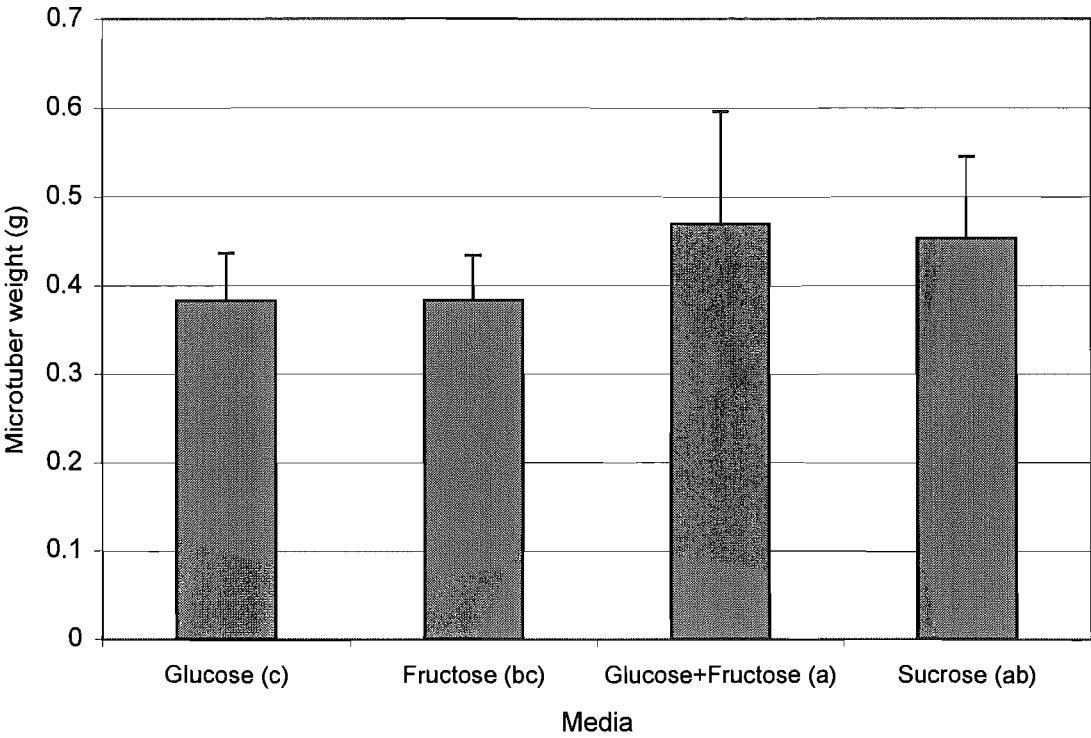


Fig. 22. Average microtuber fresh weight in response to media osmotically equivalent to the 8% sucrose medium at the beginning of microtuberization step ('Daeji'). Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.01 level.

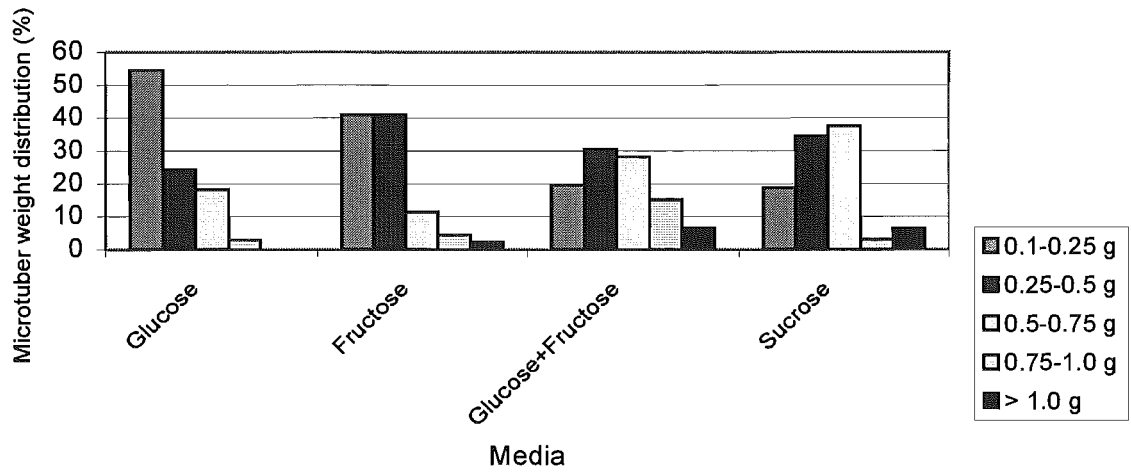


Fig. 23. Fresh microtuber weight distribution in response to media osmotically equivalent to the 8% sucrose medium at the beginning of microtuberization step ('Iwa').

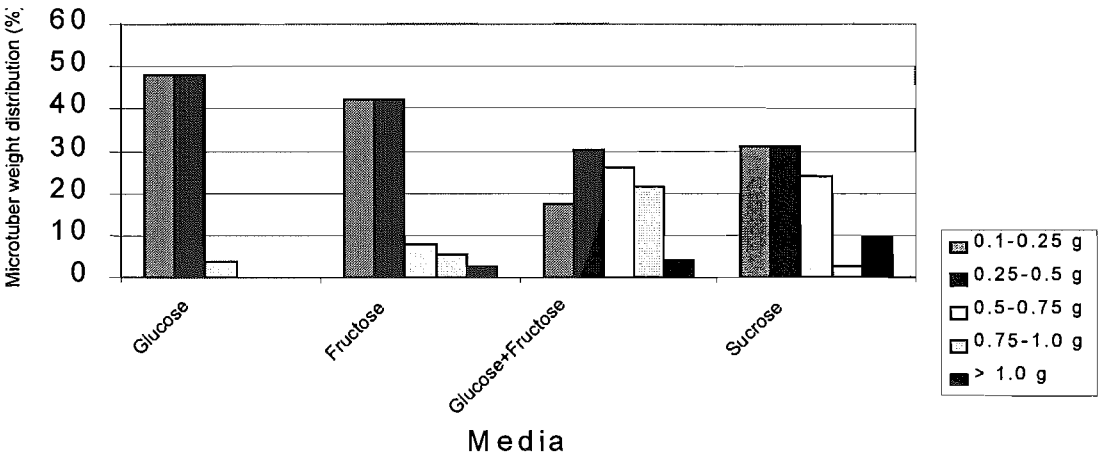


Fig. 24. Fresh microtuber weight distribution in response to media osmotically equivalent to the 8% sucrose medium at the beginning of microtuberization step ('Daeji').

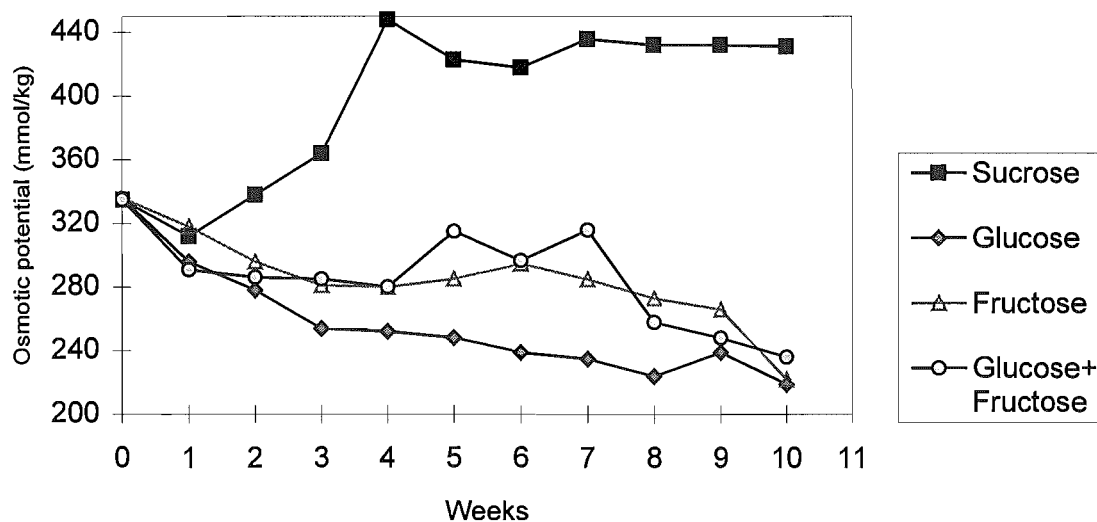


Fig. 25. Osmotic potential changes of media that were osmotically equivalent to the 8% sucrose medium at the beginning of the microtuberization step ('Iwa').

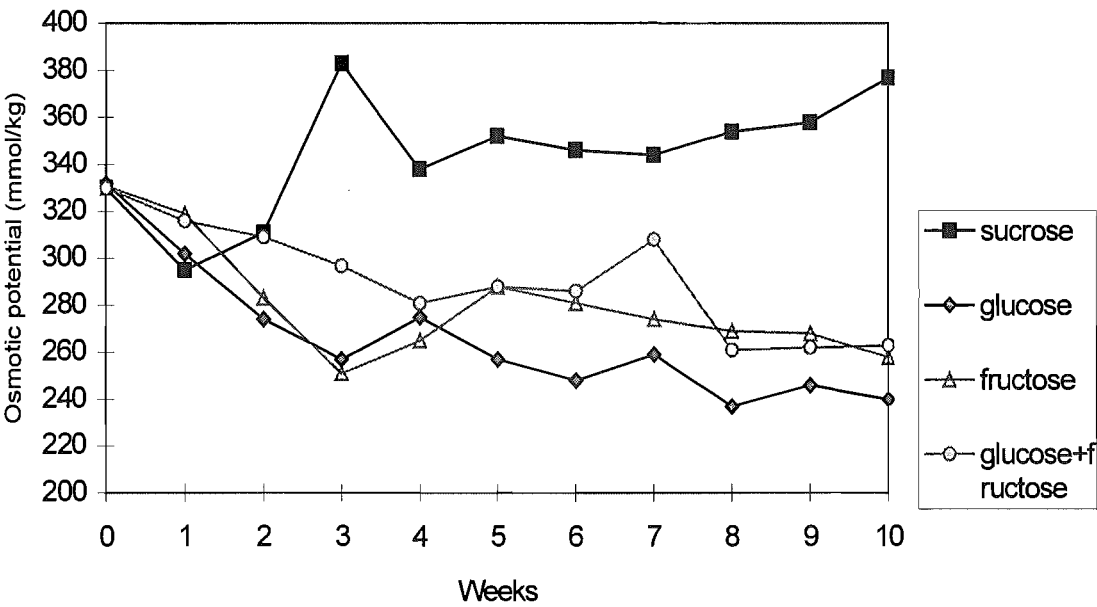


Fig. 26. Osmotic potential changes of media that were osmotically equivalent to the 8% sucrose medium at the beginning of the microtuberization step ('Daeji').

microtuberization experiment (Fig. 25), that of the glucose medium continued to decline to a level substantially lower than that of the sucrose medium in the same culture period.

Although the fructose or fructose + glucose medium ended up with the similarly low levels of osmotic potential as the glucose medium, for much of the culture period, the osmotic potential of these two former media was higher than that of the glucose medium but was still substantially lower than that of the sucrose medium. Within the first 4 weeks of the microtuberization experiment with ‘Daeji’ plantlets, similar changes in the osmotic potential levels of the four media were observed (Fig. 26). The osmotic potential of the glucose medium was at lower levels than the fructose or a mixture of glucose + fructose medium from week 5 to the end of the microtuberization experiment. During the same culture period, the sucrose medium had higher osmotic potential levels than the other three media (Fig. 26).

1.3.1.3 pH of the media changed during microtuber formation

Time course of pH changes in the media (1.3.1.1) with equivalent osmolarity at the beginning of the microtuberization step were measured to see any correlation with microtuber formation. Despite the different carbohydrates, the patterns of pH changes in the four media were similar during microtuberization experiments with ‘Iwa’ (Fig. 27) and ‘Daeji’ plantlets (Fig. 28). Overall the pH seemed to dip during the first 4 weeks, and then it went back up to pH nearly 5.5 by week 10.

1.3.2 The effect of monosaccharide-containing media with carbon content that was initially equivalent to that of 8% sucrose

1.3.2.1 Microtuber formation

Microtubers were initiated in the plantlets of both varieties ‘Iwa’ and ‘Daeji’ 2 weeks after transfer from the standard multiplication medium to the three different monosaccharide-containing media with carbon content that was initially equivalent to that of the 8% sucrose medium, in darkness. The number of microtubers formed was largely similar (about 3 per 3 plantlets) among the 4 different media (Table 4).

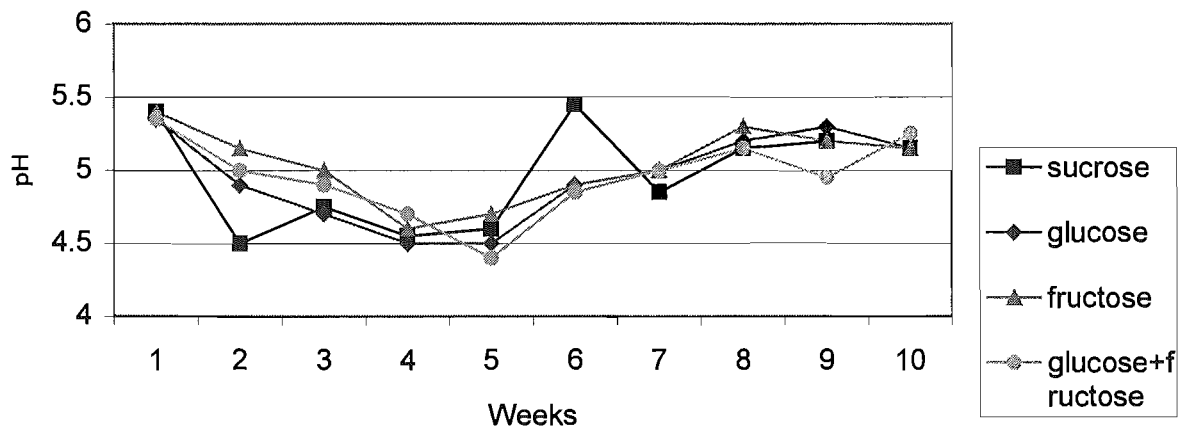


Fig. 27. Time course of pH changes in media containing monosaccharides that were osmotically equivalent to the 8% sucrose medium at the beginning of microtuberization step ('Iwa').

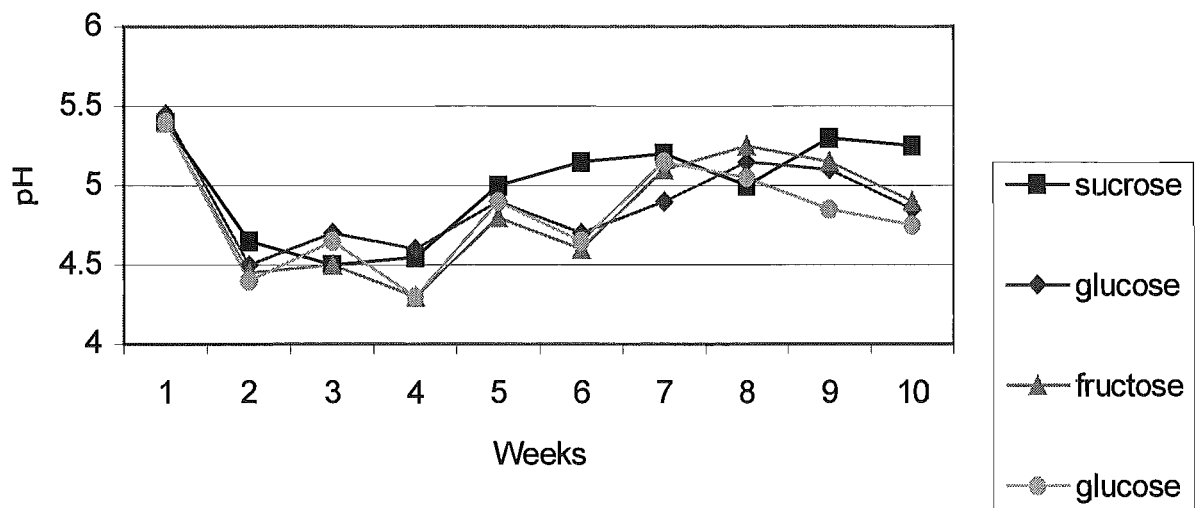


Fig. 28. Time course of pH changes in media containing monosaccharides that were osmotically equivalent to 8% sucrose medium at the beginning of microtuberization step ('Daeji').

Table 4. Effect of media containing carbon contents that were initially equivalent to that of the 8% sucrose on number of microtubers formed after 10 weeks in the dark.

Microtuber numbers produced per 3 plantlets per jar

Media	‘Iwa’	‘Daeji’
Glucose	2.60 ± 0.25	2.46 ± 1.12
Fructose	3.01 ± 1.38	2.92 ± 0.57
Glucose+Fructose	3.42 ± 1.03	2.92 ± 1.45
Sucrose	2.75 ± 0.75	3.23 ± 0.52

No significant difference was found among the treatments (ANOVA, p≤0.05)

The 'Iwa' plantlets appeared to produce microtubers of similar size to those of 'Daeji' in response to the different media initially containing monosaccharides with carbon content equivalent to that of 8% sucrose in the media (Figs. 29 and 30).

The potato plantlets seemed to respond better to the medium initially supplied with a mixture of glucose and fructose or the standard 8% sucrose microtuberization medium than in media with glucose or fructose supplied singly, although the statistical analysis only supported this in the case of 'Iwa'.

From an examination of the frequencies of microtubers in different weight categories, it is clear that despite having the same carbon content at least initially the tuberization medium containing a mixture of glucose and fructose or the standard 8% sucrose medium rather than medium containing either glucose or fructose tended to favour the production of larger microtubers in both varieties (Figs. 31 and 32). Thus, for example, the frequency of microtubers heavier than 1.0 g was 0 in the case of single monosaccharide media, but was 13%, when the 'Daeji' plantlets were cultured in the medium initially supplied with glucose and fructose (Fig. 32).

1.3.2.2 Osmotic potential changes in the different microtuberization media used in 1.3.2

The initial osmotic potential of the monosaccharide media was much higher than that of 8% sucrose medium (Figs. 33 and 34). During the first 4 weeks of the microtuberization of the 'Iwa' plantlets, the osmotic potential of the sucrose medium increased while that of the other media decreased (Fig. 33). Then the osmotic potential of the sucrose medium decreased slightly before stabilizing at a relatively high level till the end of the microtuberization step. From week 5, the osmotic potential of the fructose medium was virtually the same as that of the sucrose medium in the same time period. The osmotic potential of the glucose medium continued to decline to levels lower than that of the sucrose or fructose media in the same culture period. The osmotic potential of the mixed glucose and fructose medium remained at the same levels but higher than other media from week 4 to week 7 before dropping sharply to a level close to that of other media.

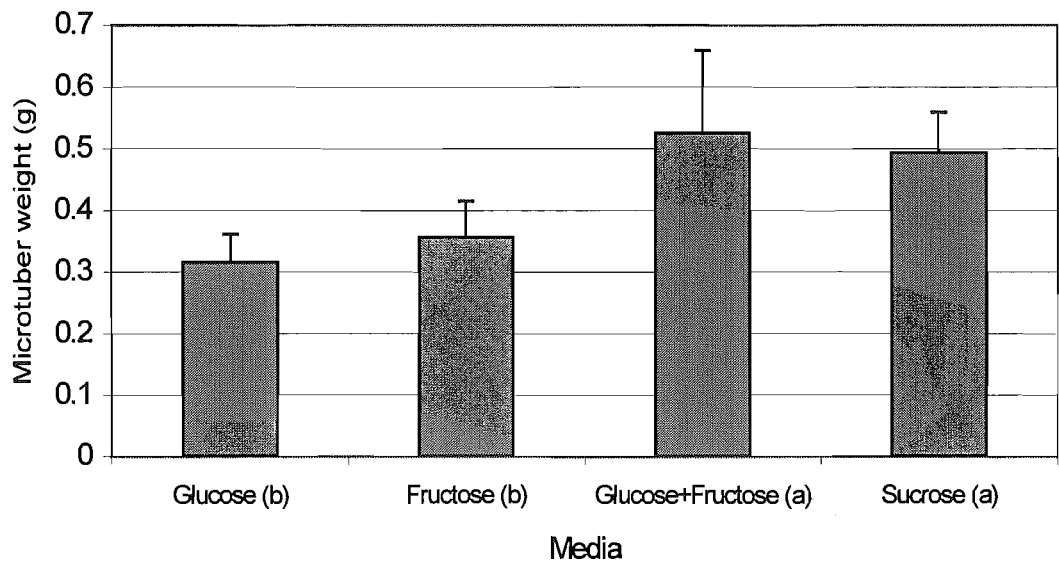


Fig. 29. Average microtuber fresh weight in response to media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of the microtuberization step ('Iwa'). Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.001 level.

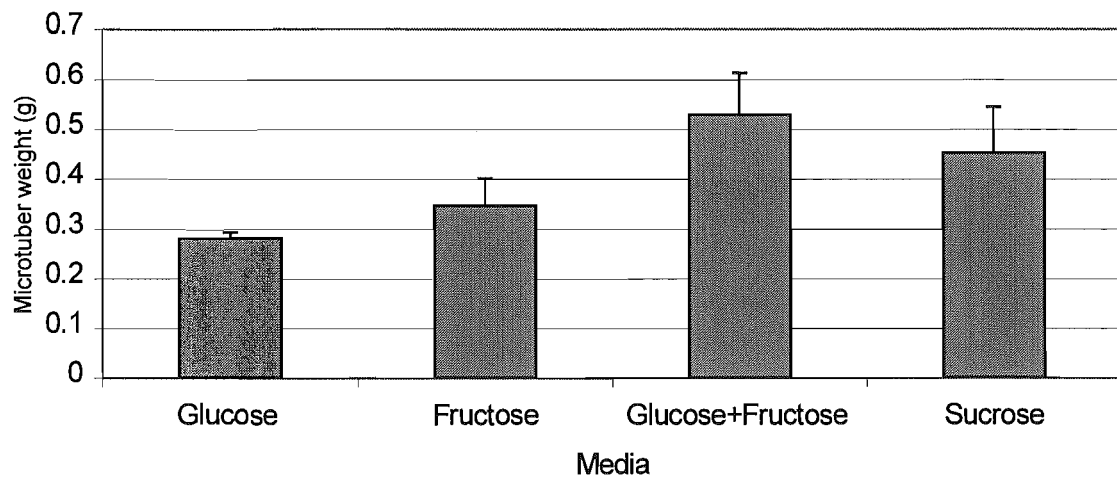


Fig. 30. Average microtuber fresh weight in response to media with carbon contents that were equivalent to the 8% sucrose medium at the beginning of the microtuberization step ('Daeji'). No significant difference was found among the treatments (ANOVA, $p \leq 0.05$)

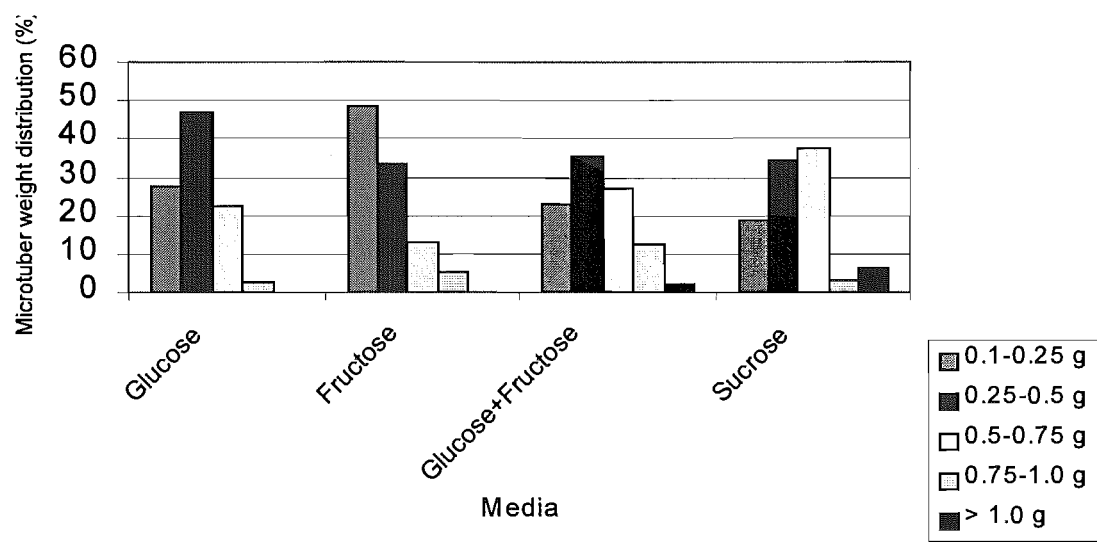


Fig. 31. Microtuber fresh weight distribution in response to media with carbon contents that were equivalent to the 8% sucrose medium at the beginning of the microtuberization step ('Iwa').

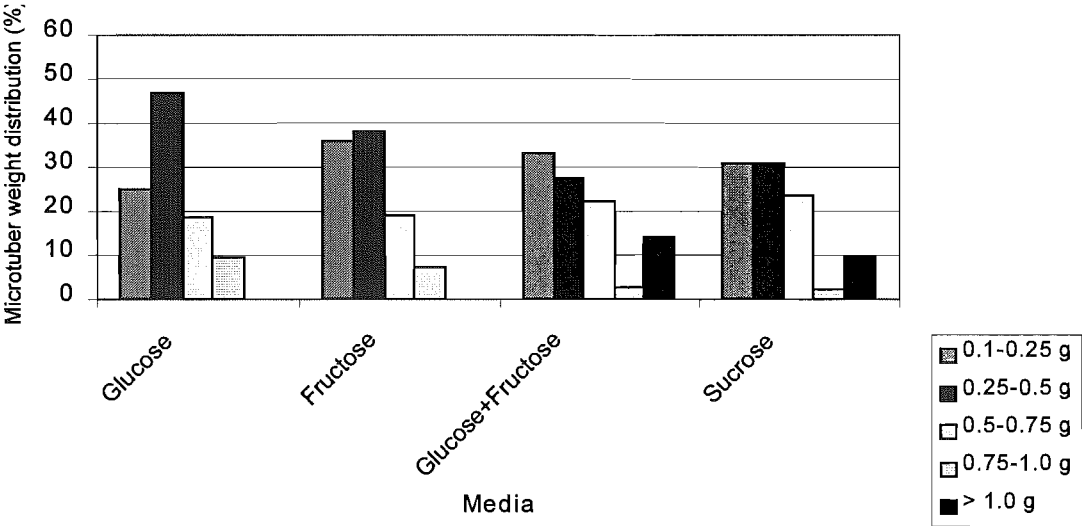


Fig. 32. Microtuber fresh weight distribution in response to media with carbon contents that were equivalent to the 8% sucrose medium at the beginning of the microtuberization step ('Daeji').

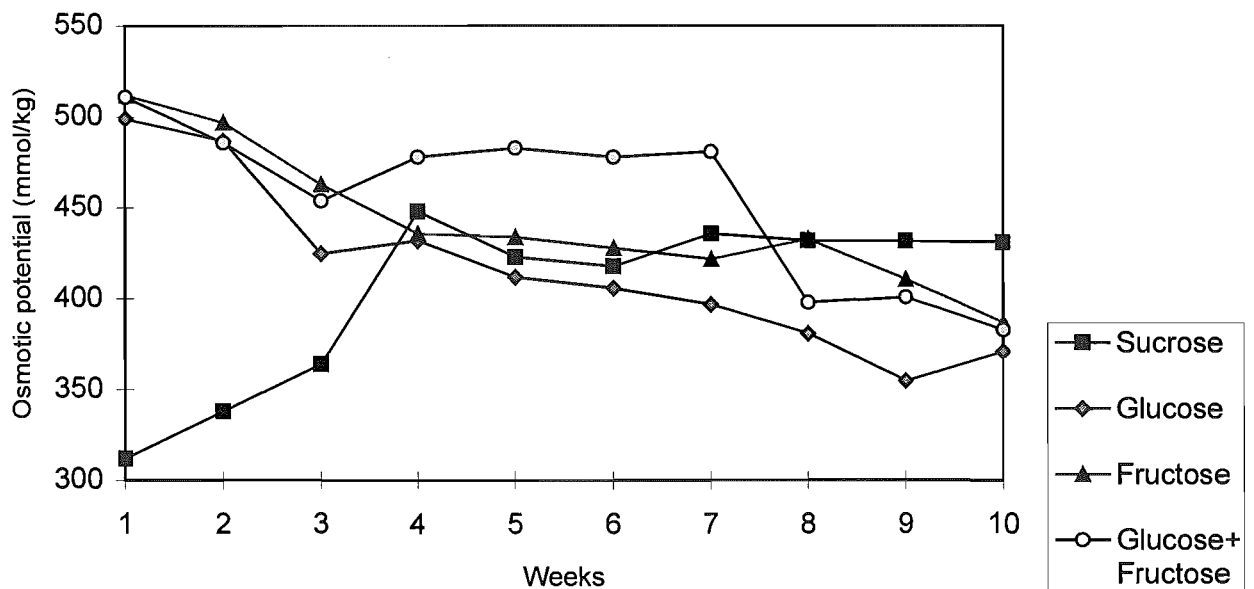


Fig. 33. Osmotic potential changes of media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Iwa').

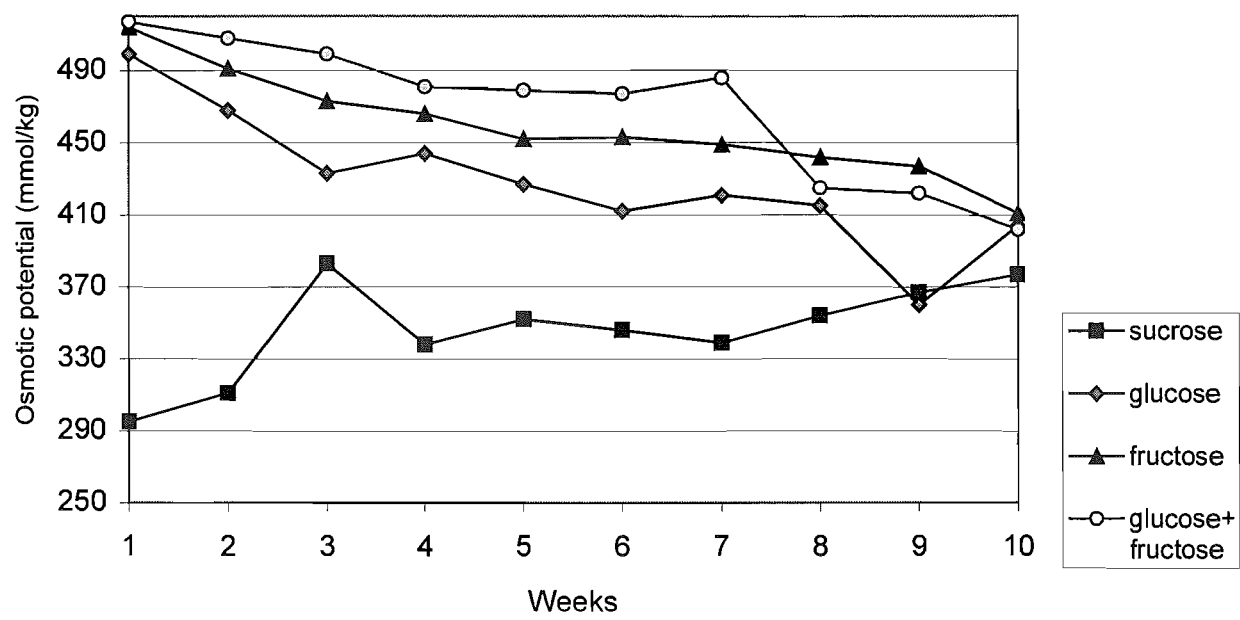


Fig. 34. Osmotic potential changes of media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Daeji').

One notable difference between the monosaccharide and the sucrose media, particularly during *in vitro* tuberization of 'Daeji' plantlets, was that the osmotic potential of the former remained higher than that of the latter throughout much of the microtuberization step (Fig. 34).

1.3.2.3 pH of the media used in 1.3.2 changed during microtuberization

The possible relationship between the time course of pH changes and tuber growth was studied. There was a gradual decline in the pH of the media during the first 2-4 weeks, and then the pH rose gradually to a level similar to that at the start of microtuberization (Figs. 35 and 36). Despite of the presence of different carbohydrates, there was no significant difference in pH changes among the different media and the overall trends were similar in 'Iwa' and 'Daeji'.

1.3.3 Substitution of sucrose with maltose

1.3.3.1 Effect on microtuberization

Following 4 weeks of the standard plantlet multiplication protocol, the effects of culturing the plantlets in media containing 8% maltose which were osmotically equivalent to 8% sucrose on the number of microtubers formed, their average fresh weights and relative size distribution were investigated.

Microtubers were initiated in the plantlets of both varieties 'Iwa' and 'Daeji' 2 weeks after transfer from the multiplication medium to either sucrose or maltose medium. The number of microtubers formed was similar (about 3 per 3 plantlets) in both treatments (Table 5a). The 'Iwa' plantlets appeared to form microtubers of similar size in response to the two osmotically equivalent media (Table 5b), but they produced bigger microtubers than 'Daeji' plantlets in response to the 8% maltose medium. Both varieties responded similarly in the 8% sucrose medium.

From an examination of the frequencies of microtubers in different weight categories, it is clear that 'Iwa' plantlets tended to produce similar size distributions of microtubers in both disaccharide media (Fig. 37). However, 'Daeji' plantlets appeared to react to the maltose medium by producing more microtubers in 0.1 to 0.25g category when compared to the

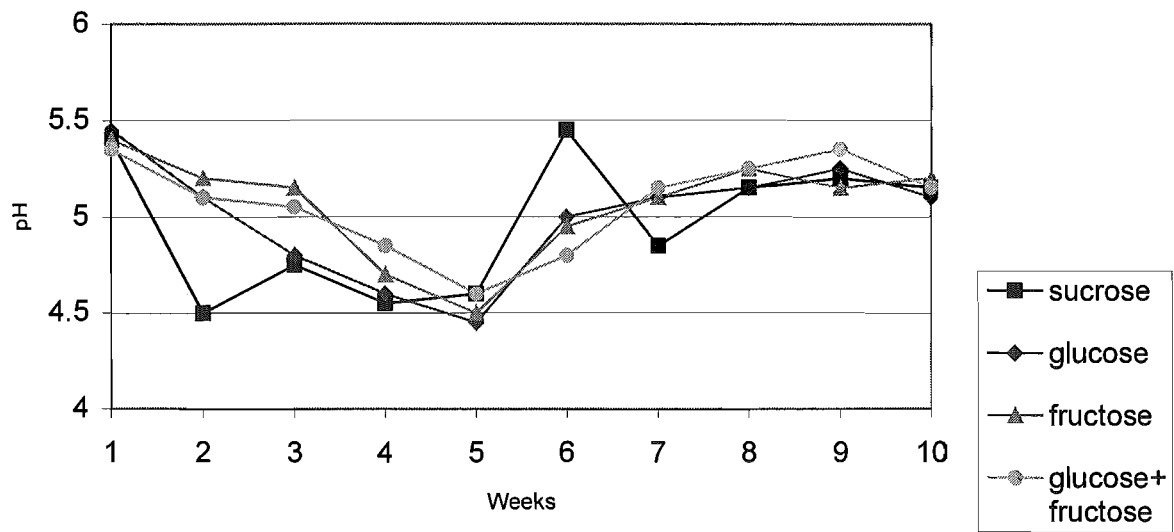


Fig. 35. Time course of pH changes in response to monosaccharide-containing media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Iwa').

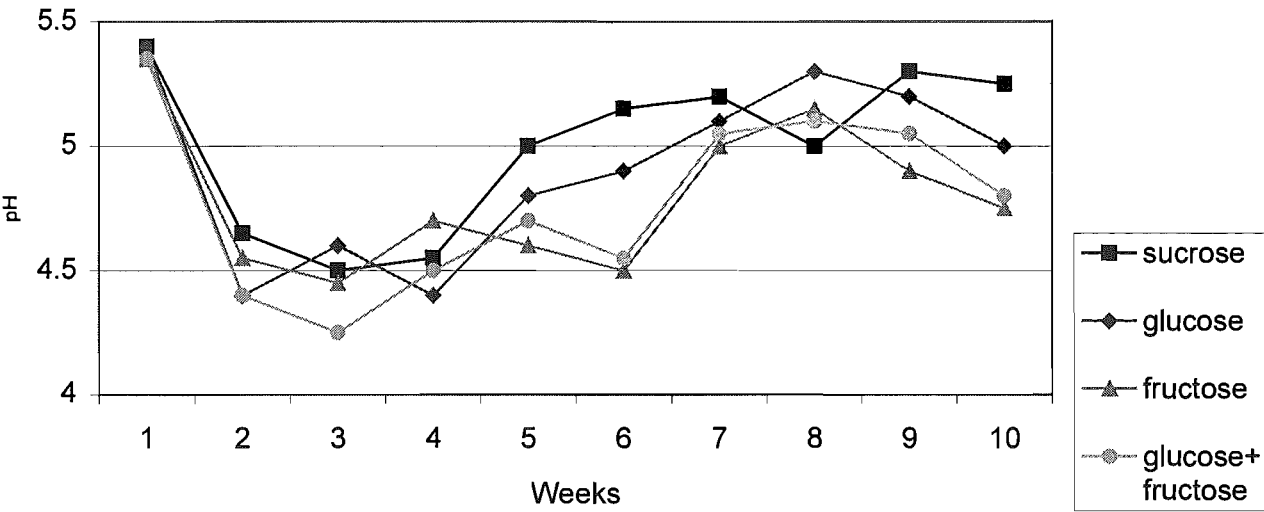


Fig. 36. Time course of pH changes in response to monosaccharide-containing media with carbon contents that were equivalent to that of the 8% sucrose medium at the beginning of microtuberization step ('Daeji').

Table 5a. Effect of maltose and sucrose on microtuber number (a) and average microtuber weight (b).

(a) Microtuber number (microtubers produced per 3 plantlets per jar)

Media	‘Iwa’	‘Daeji’
Sucrose	2.75 ± 0.75	3.23 ± 0.52
Maltose	3.15 ± 0.64	3.00 ± 1.27

(b) Average microtuber weight (g)

Media	‘Iwa’	‘Daeji’
Sucrose	0.49 ± 0.06	0.45 ± 0.09
Maltose	0.54 ± 0.21	0.32 ± 0.05

No significant difference was found among the treatments (ANOVA, $p \leq 0.05$)

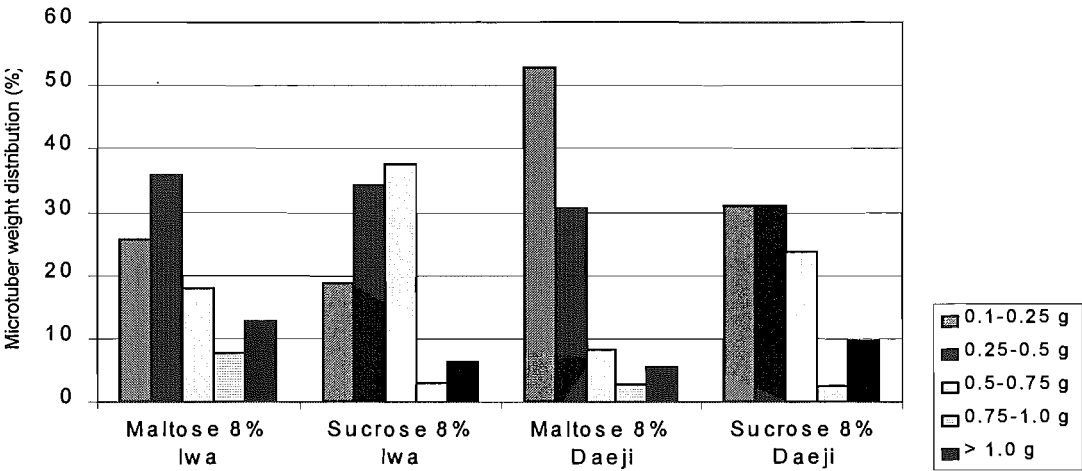


Fig. 37. Microtuber fresh weight distribution in response to media containing 8% (w/v) maltose or sucrose at the beginning of the microtuberization step ('Iwa' and 'Daeji').

sucrose medium (Fig. 38).

1.3.3.2 Osmotic potential changes of the 8% maltose medium

The initial osmotic potential of the maltose medium was the same as that of the standard 8% sucrose microtuberization medium. In the first five weeks during microtuberization of both 'Iwa' and 'Daeji' plantlets, there was little change in the osmotic potential of the maltose medium compared to the sucrose medium (Figs. 38 and 39). Then the osmotic potential of the maltose medium showed a steady decrease.

1.3.3.3 Time course of pH changes in the 8% disaccharide media

During first few weeks of microtuberization of both 'Iwa' and 'Daeji' plantlets, the pH of both the maltose and sucrose media tended to decrease to a low point around 4 - 4.5 before returning gradually in the following weeks to the level at start of microtuberization (Figs. 40 and 41). One exception was that the pH in the medium for 'Iwa' plantlets in sucrose did not change initially until week 4 (fig. 40).

1.4 Effect of initial pH of the tuberization medium

Following 4 weeks of the standard plantlet multiplication protocol, the effects of culturing the plantlets in media with an initial pH of 4.0 (low pH) or 8.0 (high pH) on the number of microtubers formed, their average fresh weights and relative size distribution were investigated.

Microtubers were initiated in the plantlets of both varieties 'Iwa' and 'Daeji' 2 weeks after transfer from the multiplication medium to media at initial pH either higher or lower than that of the standard microtuberization medium (pH 6.0). The number of microtubers formed by 'Iwa' plantlets was similar (about 3 per 3 plantlets) in high pH or (3.14 per 3 plantlets) in low pH treatments compared to 2.9 microtubers per 3 plantlets in standard medium (pH 6.0), whereas in the case of 'Daeji' about 3.5 microtubers per 3 plantlets in high pH and about 5.3 microtubers per 3 plantlets in low pH medium were observed compared to 3.1 microtubers per plantlets in standard medium (pH 6.0). The statistical analysis indicated that significantly more microtubers were initiated by 'Daeji' plantlets in

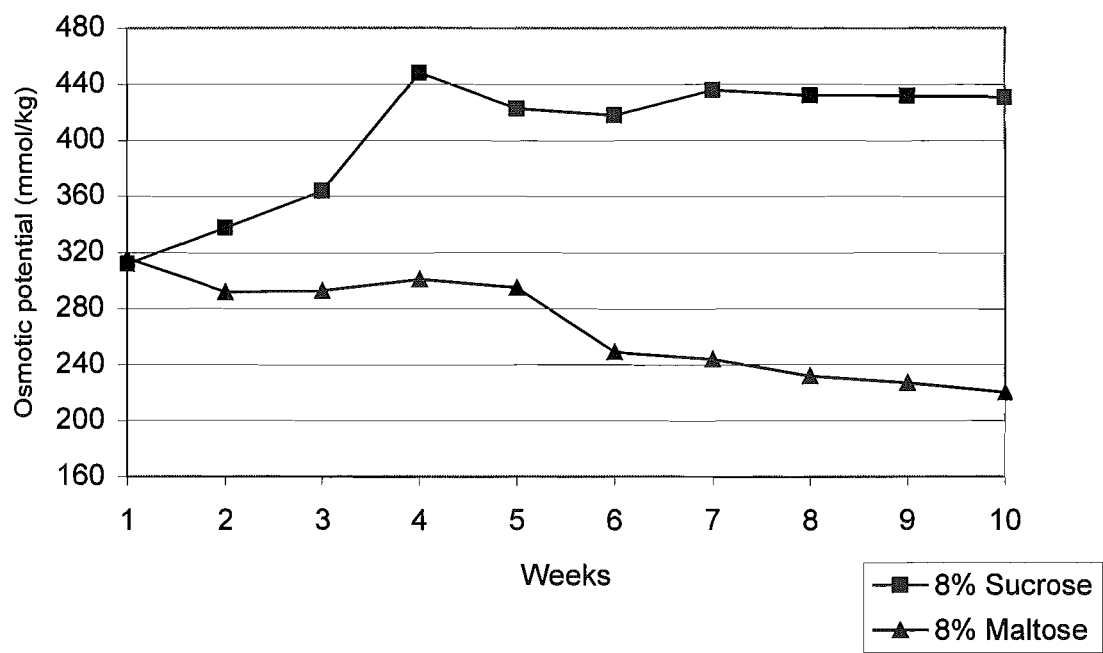


Fig. 38. Osmotic potential changes of media containing 8% (w/v) sucrose or maltose at the beginning of the microtuberization step ('Iwa').

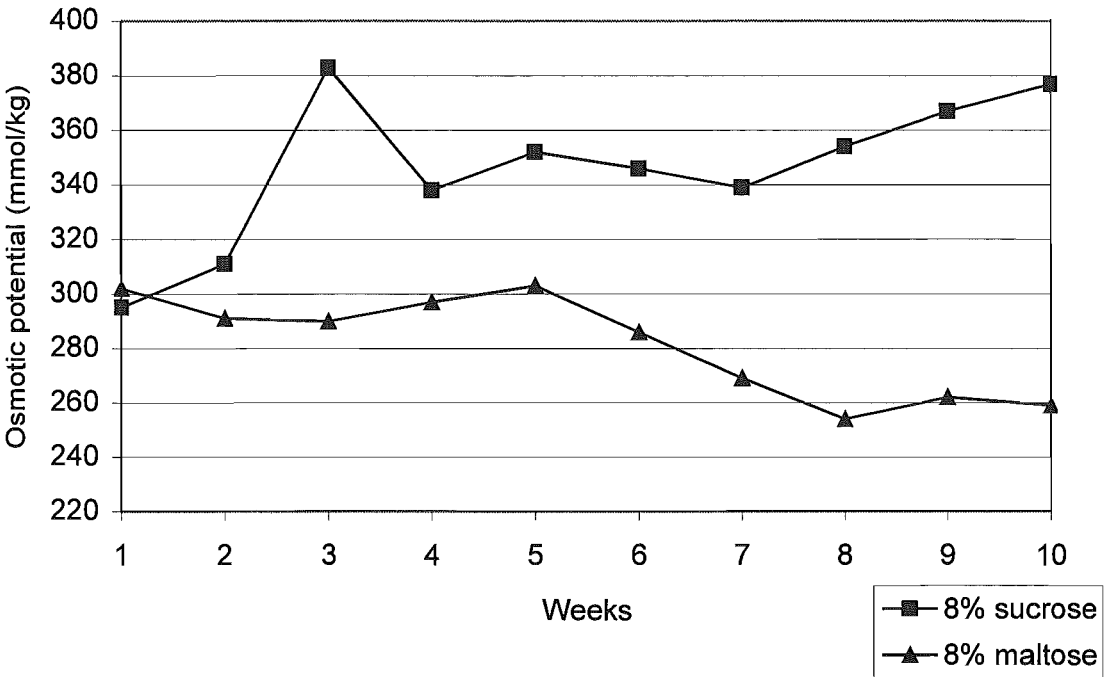


Fig. 39. Osmotic potential changes of media containing 8% (w/v) sucrose or maltose at the beginning of the microtuberization step ('Daeji').

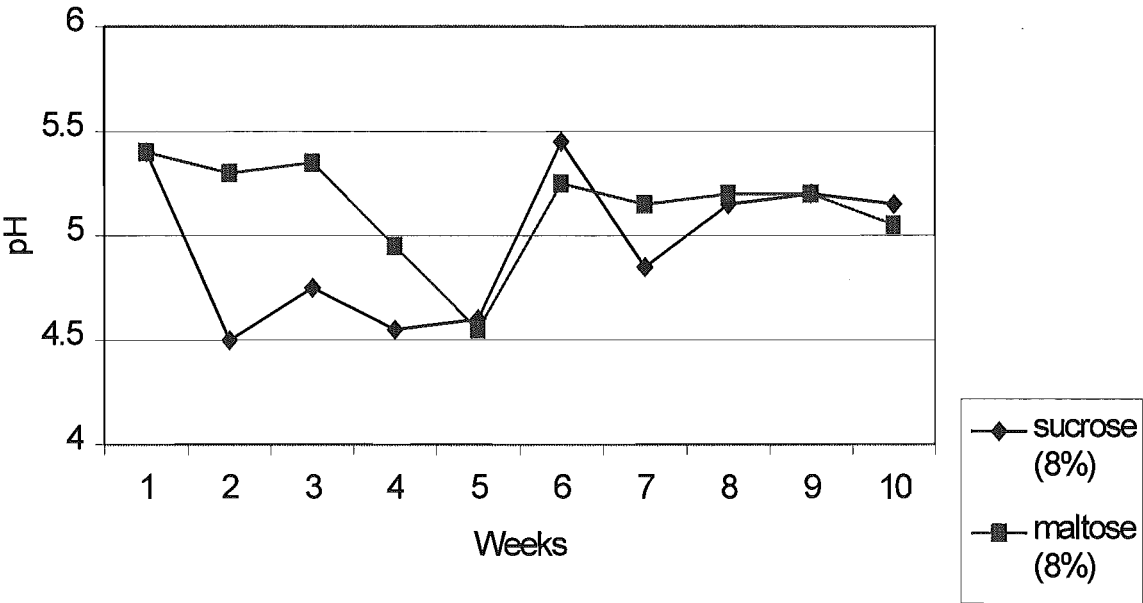


Fig. 40. pH changes on media containing 8% (w/v) sucrose or maltose at the beginning of the microtuberization step ('Iwa').

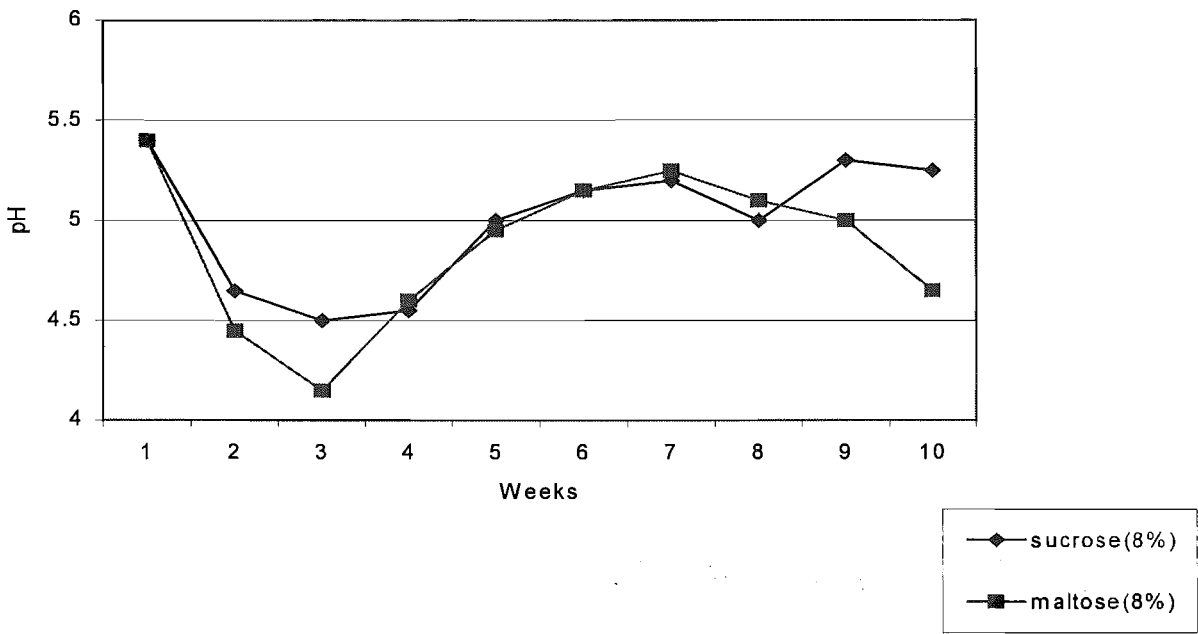


Fig. 41. pH changes on media containing 8% (w/v) sucrose or maltose at the beginning of the microtuberization step ('Daeji').

response to the low pH medium ($p \leq 0.05$).

The 'Daeji' plantlets appeared to be more sensitive to the variations of the initial pH of the tuberization than those of 'Iwa' as high or low pH treatments had adverse effects on the average weight of microtubers formed in 'Daeji', but not in 'Iwa' (Figs. 42 and 43).

The size distribution of microtubers formed by 'Iwa' plantlets was similar in all treatments despite the differences in the pH of the media at the beginning of the microtuberization step (Fig. 44). By contrast, the size distribution of microtubers formed by 'Daeji' plantlets was adversely affected by the high or low pH medium (i.e. more smaller tubers formed than in the standard tuberization medium), particularly when the pH of the medium was high at the onset of the microtuberization step (Fig. 45).

1.5 Effect of old multiplication medium mixed with tuberization medium

Following 4 weeks of the standard plantlet multiplication protocol, the multiplication medium was retained and then fresh microtuberization medium with varying concentrations of sucrose ranging from 4 to 20% (w/v) was added. The actual sucrose content in each jar was lowered following addition of the appropriate sucrose-containing tuberization medium, as there was a $20 \pm 3\%$ increase in combined volume of both media. The number of microtubers formed, their average fresh weights and relative size distribution were recorded after 10 weeks of culture.

Microtubers were initiated in the plantlets of both varieties 'Iwa' and 'Daeji' within 2 weeks of transfer from the multiplication medium to a wide range of sucrose-containing new media mixed with the retained old multiplication medium. The number of microtubers formed was similar (about 3.5 per 3 plantlets) when 'Iwa' plantlets were cultured in most sucrose treatments (Table 6) but was slightly lower (about 2.9 per 3 plantlets) in the 16% sucrose medium. The 'Daeji' plantlets reacted differently to the different mixed media as more microtubers were formed (Table 6) in response to the '20%' sucrose medium.

A reduction in microtuber size was observed when 'Iwa' or 'Daeji' plantlets were cultured in media other than '12%' sucrose medium (Figs. 46 and 47).

From an examination of the frequencies of microtubers in different weight categories, it is

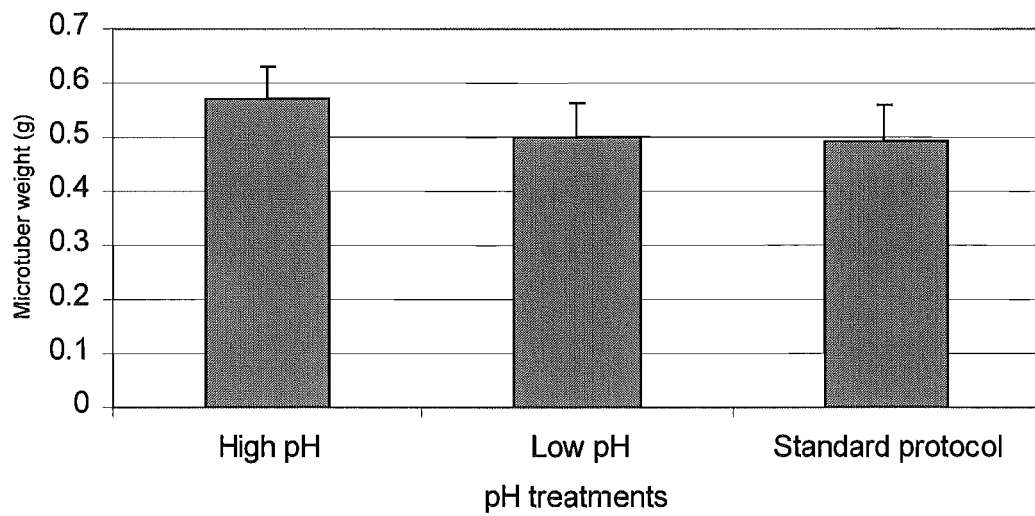


Fig. 42. Average fresh weight of microtubers in response to the initial pH of the tuberization medium ('Iwa'). High pH= pH 8.0; Low pH= pH 4.0 and standard protocol= pH 6.0. No significant difference was found among the treatments (ANOVA, $p \leq 0.05$)

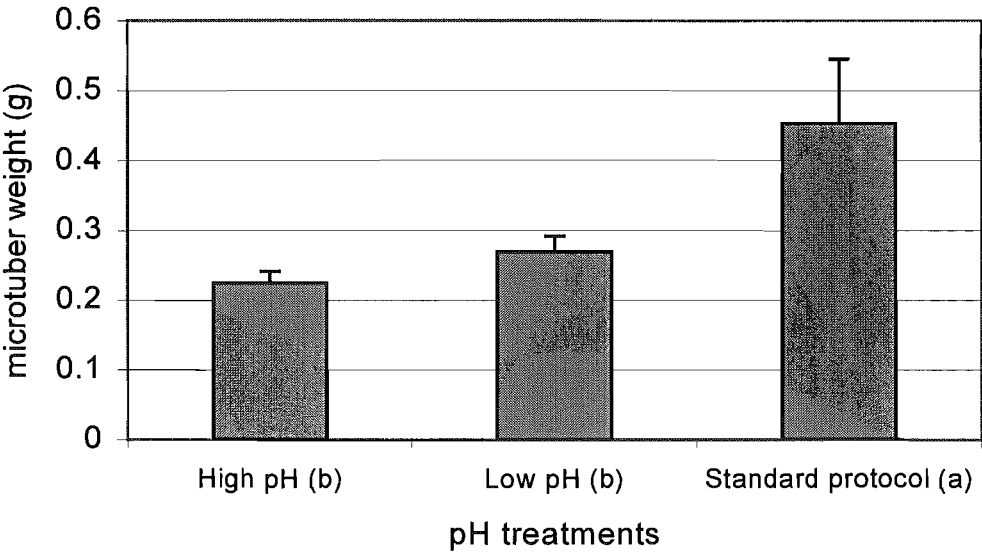


Fig. 43. Average fresh weight of microtubers in response to the initial pH of the tuberization medium ('Daeji'). High pH= pH 8.0; Low pH= pH 4.0 and standard protocol= pH 6.0. Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.01 level.

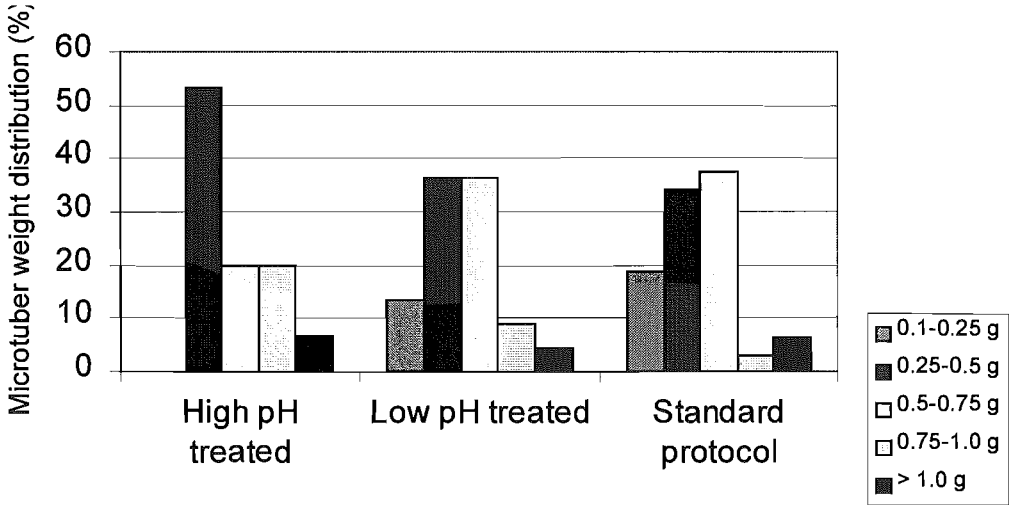


Fig. 44. Microtuber fresh weight distribution in response to the initial pH of the tuberization medium ('Iwa').

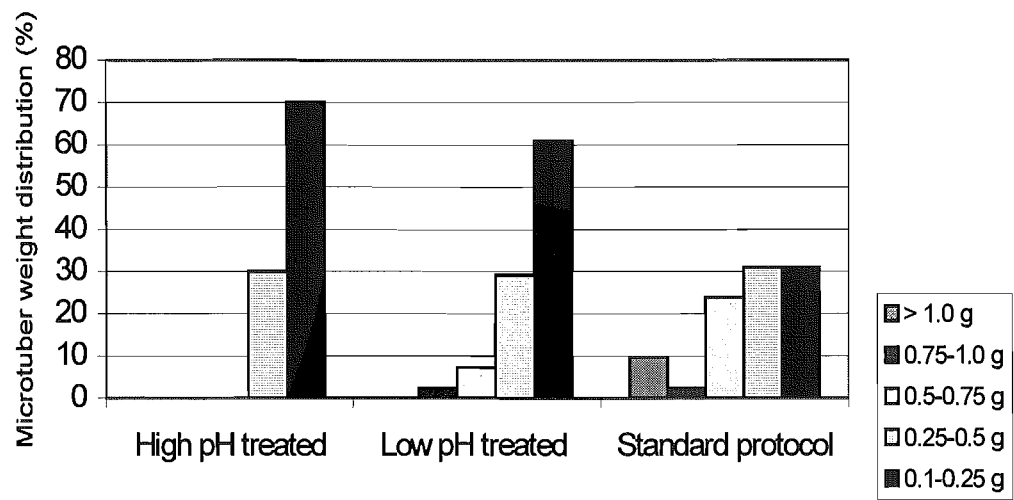


Fig. 45. Microtuber fresh weight distribution in response to the initial pH of the tuberization medium (‘Daeji’).

Table 6. Effect of old multiplication medium mixed with fresh tuberization medium.

(Number of microtubers produced per 3 plantlets per jar)

Media	'Iwa'	'Daeji'
4% Sucrose	3.71 ± 0.57	3.66 ± 0.26
8% Sucrose	3.60 ± 1.60	2.66 ± 1.50
12% Sucrose	3.60 ± 1.37	2.70 ± 1.78
16% Sucrose	2.88 ± 0.61	4.33 ± 0.33
20% Sucrose	2.70 ± 1.34	4.88 ± 1.86

No significant difference was found among the treatments (ANOVA, $p \leq 0.05$)

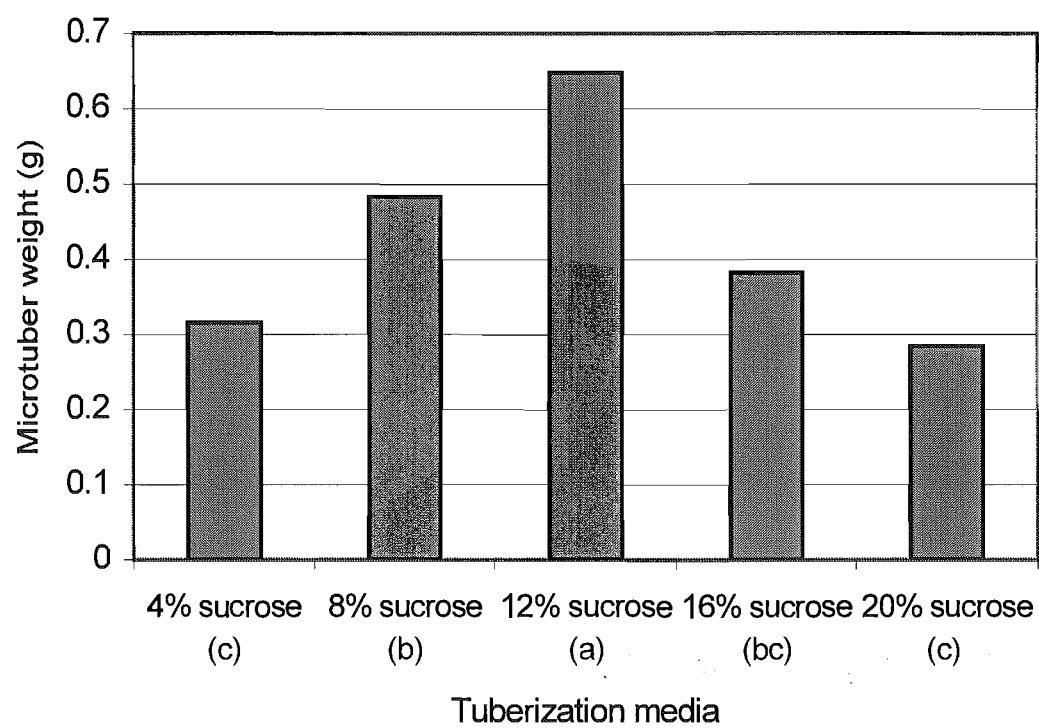


Fig. 46. Effect of mixing old multiplication medium with fresh tuberization medium on average microtuber fresh weight ('Iwa'). Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.001 level.

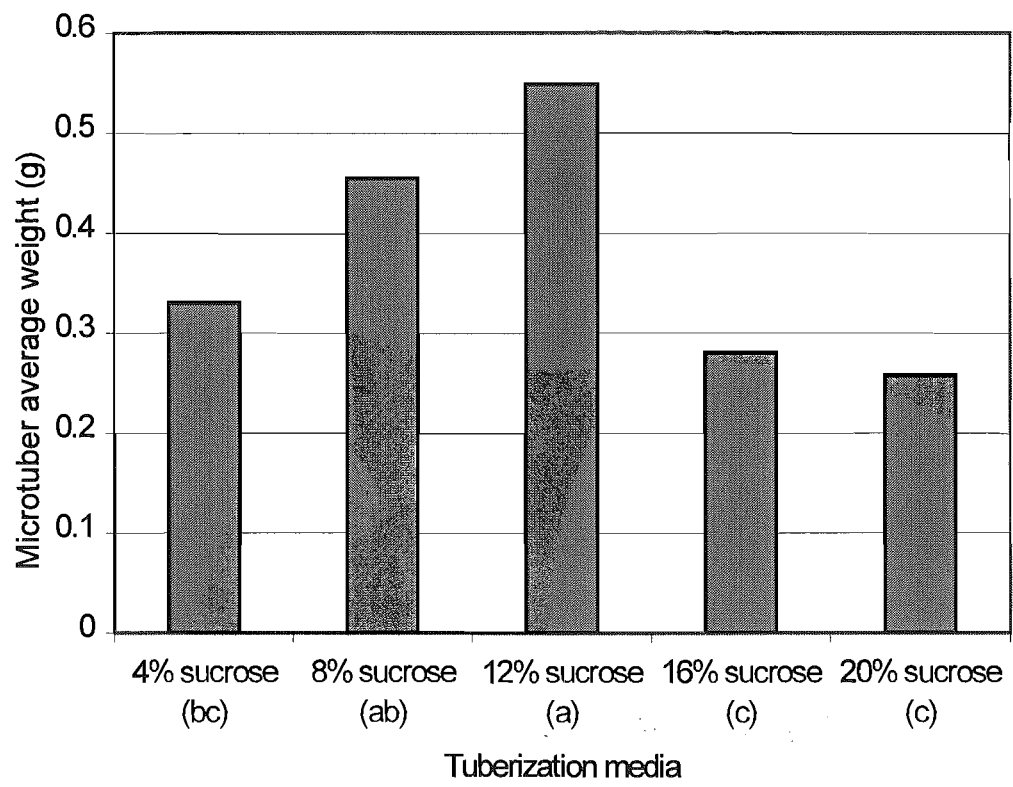


Fig. 47. Effect of mixing old multiplication medium mixed with fresh tuberization medium on average fresh microtuber weight ('Daeji'). Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.001 level.

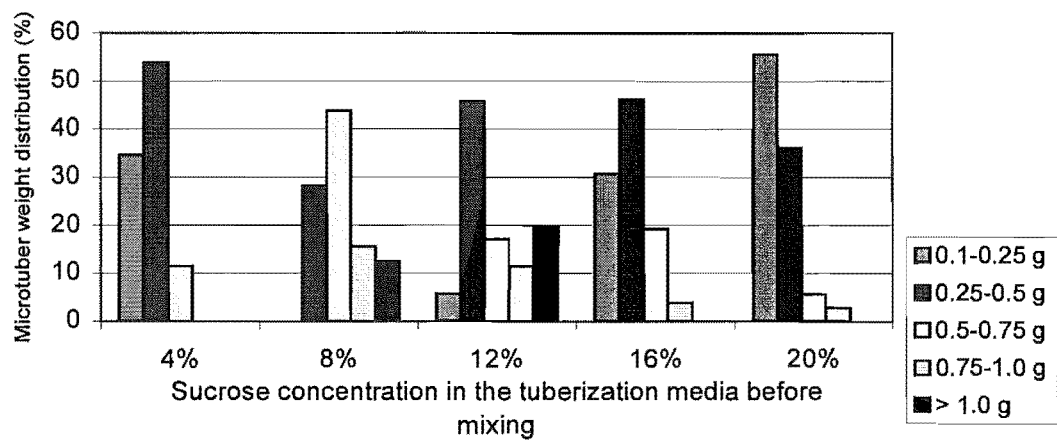


Fig. 48. Effect of mixing old multiplication medium with fresh tuberization medium on microtuber weight distribution ('Iwa').

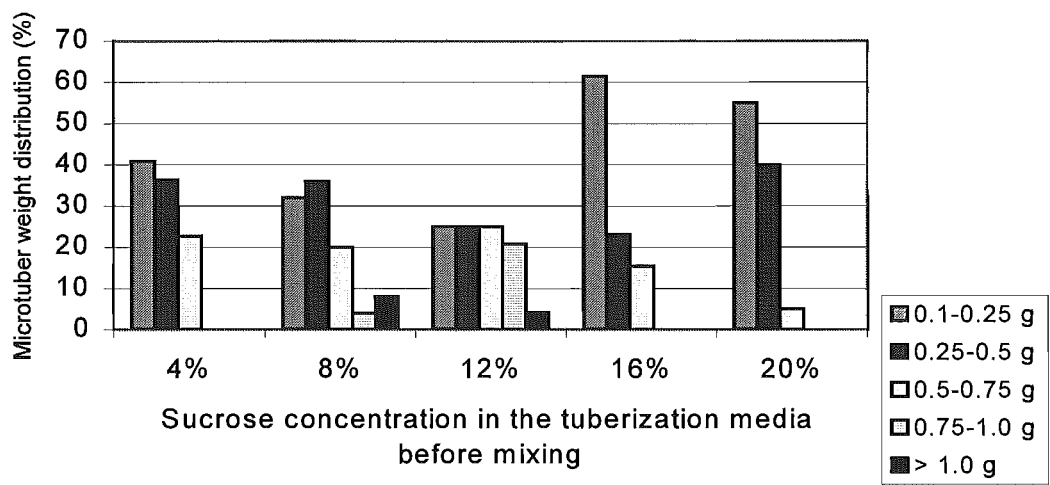


Fig. 49. Effect of mixing old multiplication medium with fresh tuberization medium on microtuber weight distribution ('Daeji').

clear that the '8%' and 12% sucrose media tended to favor the production of larger microtubers in both varieties (Figs. 48 and 49). In particular, culturing in the '20%' sucrose medium led predominantly to the production of smaller tubers, and microtubers heavier than 1.0 g were not formed in the case of 4, 16, 20% sucrose media.

1.6 Effect of periodically refreshing the standard tuberization medium

The standard tuberization medium was discarded and replaced with fresh medium of the same composition at 1, 2, 3, 4, 5 week intervals. All cultures were harvested at week 10, and the number of microtubers formed, their average fresh weights and relative size distribution were determined. In both 'Iwa' and 'Daeji' replacing medium, particularly every 1 or 2 weeks, tended to increase the number of microtubers formed compared to the standard microtuberization protocol without medium change for the entire 10 weeks (Figs. 50 and 51). Also the effectiveness of replacing the medium in promoting the number of microtubers formed seemed to decrease with a decrease in the frequency of medium replacement.

Overall, replacing the medium during microtuberization appeared to have a positive effect on the average microtuber weight achieved compared to the standard microtuberization protocol. Both varieties displayed remarkably similar responses to the medium replacement manipulation (Figs. 52 and 53). In contrast to the effect on microtuber number, replacing the medium after 3 weeks rather than after 1 or 2 weeks seemed to have a significant positive effect on the average microtuber weight at least in the case of 'Iwa' plantlets (Fig. 52).

From an examination of the frequencies of microtubers in different weight categories (Figs. 54 and 55), it can be seen that medium replacement do not appear to have a substantial effect on the size distribution of microtubers formed.

A major phenotypic change resulted from medium replacements as longitudinal shaped microtubers were observed in most cases of replacement compared to round shaped microtubers observed in the standard tuberization treatment (Fig. 56).

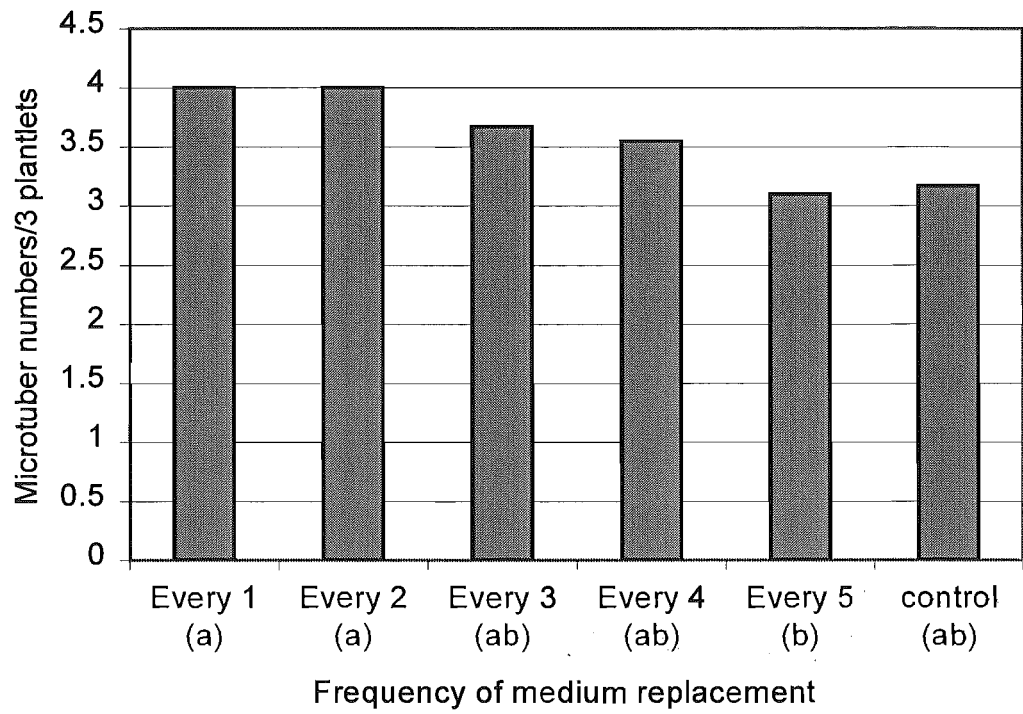


Fig. 50. Effect of medium replacement on the number of microtubers formed ('Iwa').

Control= no medium change for entire 10 weeks of the microtuberization step.

Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.05 level.

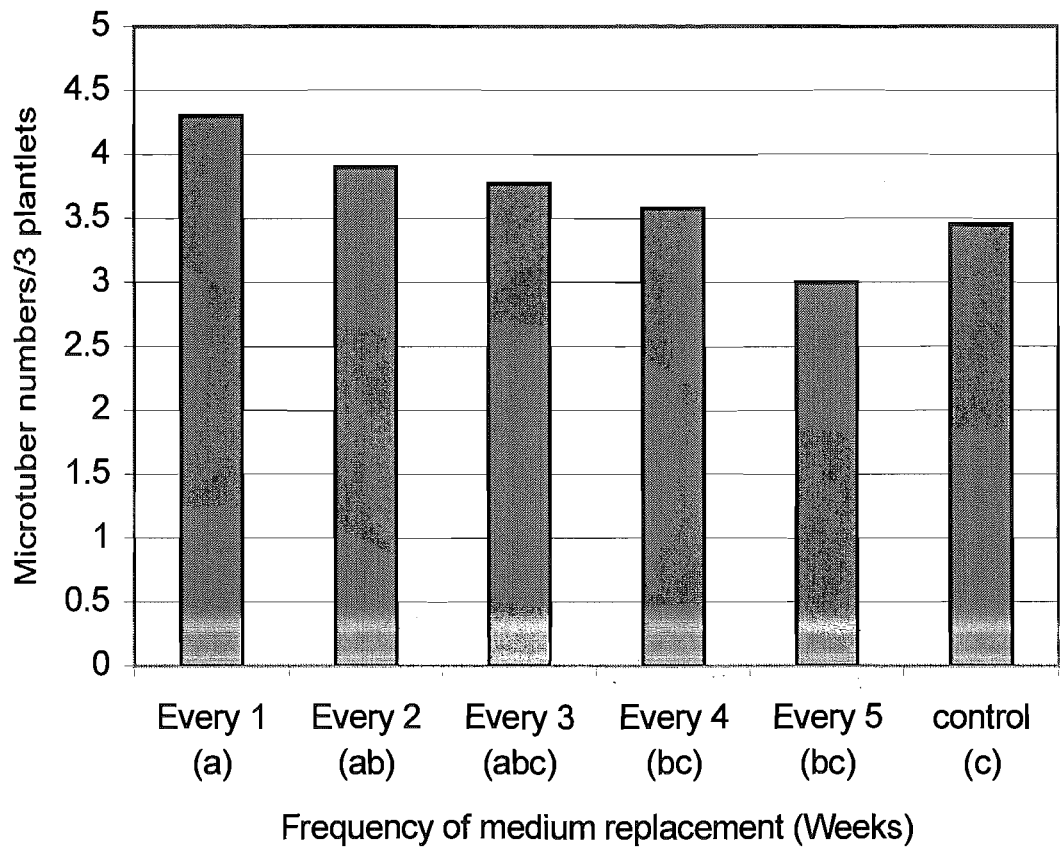


Fig. 51. Effect of medium replacement on number of microtubers formed ('Daeji').
Control= no medium change for entire 10 weeks of the microtuberization step.
Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.05 level.

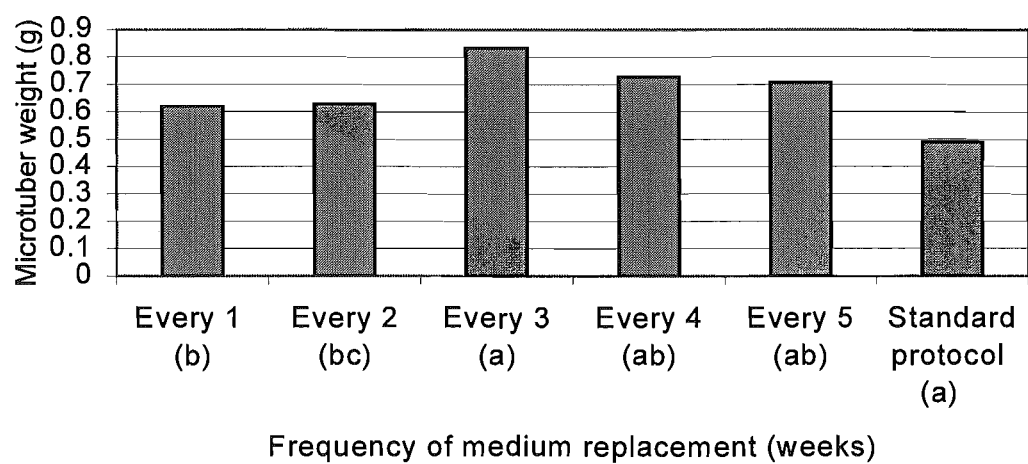


Fig. 52. Effect of periodical medium replacement on average fresh weight of microtubers ('Iwa'). Standard protocol= no replacement. Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.01 level.

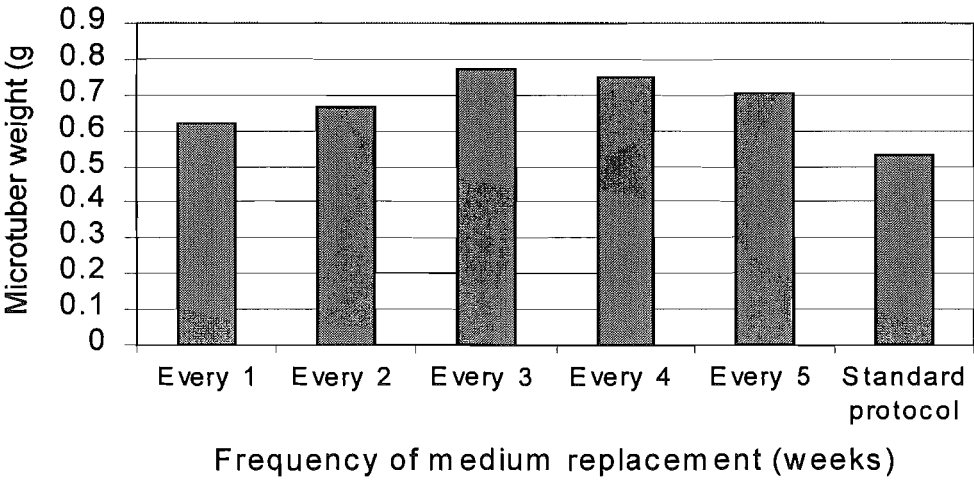


Fig. 53. Effect of periodical medium replacement on average fresh weight of microtubers ('Daeji'). Standard protocol= no replacement. No significant difference was found among the treatments (ANOVA, $p \leq 0.05$)

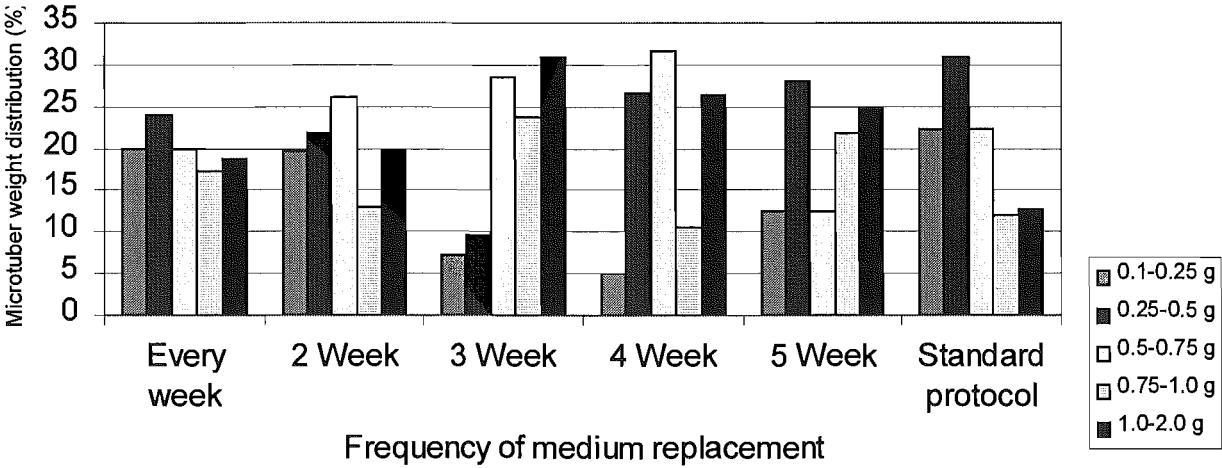


Fig. 54. Effect of medium replacement on fresh microtuber weight distribution ('Iwa').
Standard protocol= no replacement.

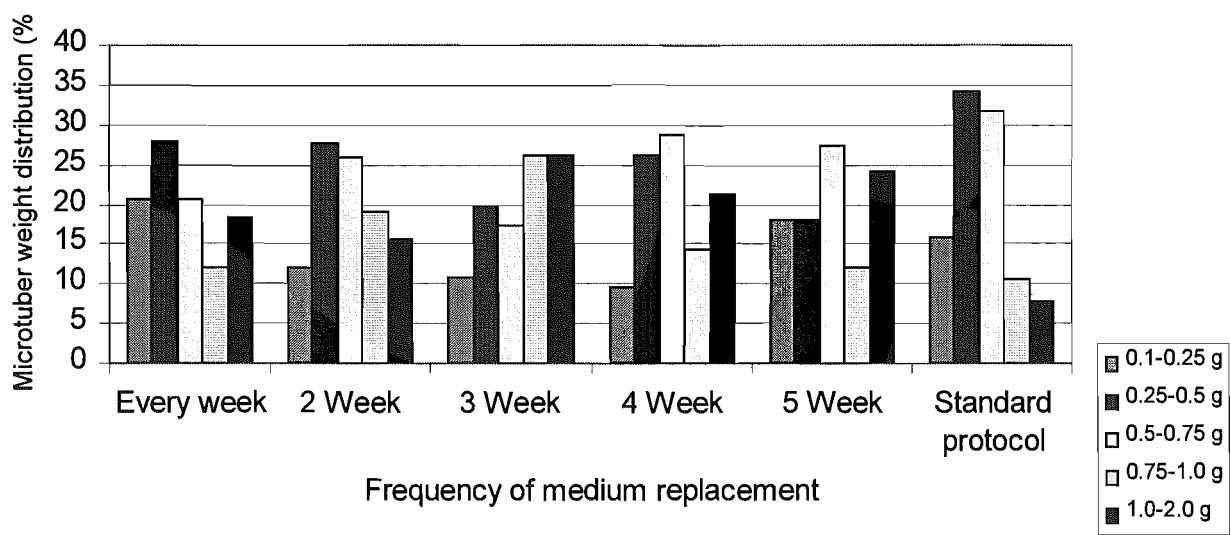


Fig. 55. Effect of medium replacement on fresh microtuber weight distribution (‘Daeji’).
Standard protocol= no replacement.



Fig. 56. A longitudinal shaped microtuber (L) obtained by periodic medium replacement treatment during microtuberization compared to a round shaped microtuber (R) by the standard tuberization protocol.

2. Manipulations during the plantlet multiplication phase: effects on the cultures during multiplication

2.1 Standard plantlet multiplication medium containing 3% sucrose

2.1.1 Plantlet development

In the stationary liquid medium, 2-nodal potato shoot explants of both 'Iwa' and 'Daeji' developed into *in vitro* plants with an upright vigorous shoot and well-developed, long green root after 4 weeks under continuous illumination. Both fresh and dry weights of the plantlets increased during culture (Figs. 57 and 58).

2.1.2 Changes in the medium during potato plantlet development

2.1.2.1 Soluble proteins

During 'Iwa' plantlet development, the concentrations of soluble proteins increased in the medium, particularly between weeks 3 and 4. This increase appeared to slow down after 4 weeks of culture (Fig. 59).

2.1.2.2 Carbohydrates

Preliminary paper chromatographic analysis of the medium revealed that the level of sucrose initially present at the beginning of culture changed during plantlet development. This was confirmed in the following HPLC analysis of the media for growing 'Iwa' and 'Daeji' plantlets (Figs. 60 and 61).

After 2 weeks of culture, glucose and fructose were detected in addition to sucrose. After 4 weeks in culture, sucrose completely disappeared from the medium with the further increase in the levels of glucose and fructose therein. The monosaccharides appeared to be present together in similar amounts in the medium.

2.1.2.3 Invertase activity in the medium

Invertase activity could be detected in the medium after 2 weeks of culture of 'Iwa' plantlets. The dependence of the enzyme activity on the pH of enzyme assays is shown in Fig. 62. A major peak of activity was detected at around pH 5.0, indicating that the sucrose-cleaving

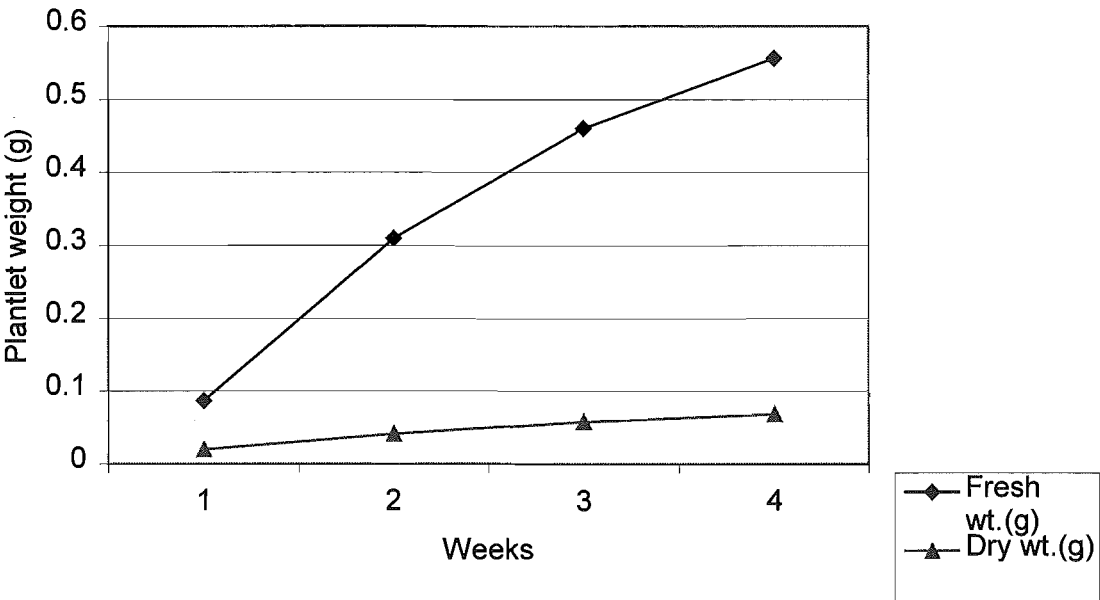


Fig. 57. Time course of changes in the fresh and dry weights per plantlet during potato plantlet development during multiplication ('Iwa').

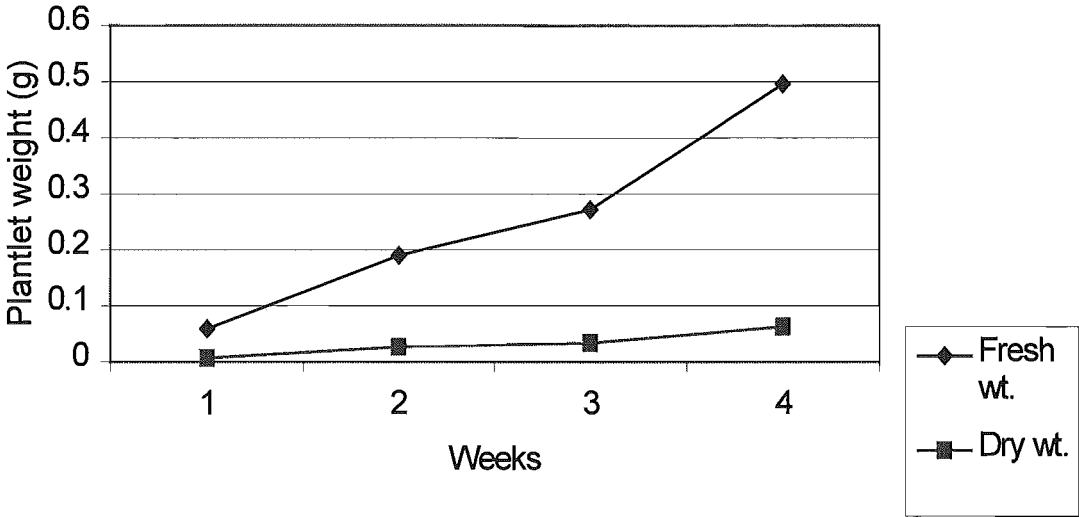


Fig. 58. Time course of increases in fresh and dry weights per plantlet during potato plantlet development during multiplication (‘Daeji’).

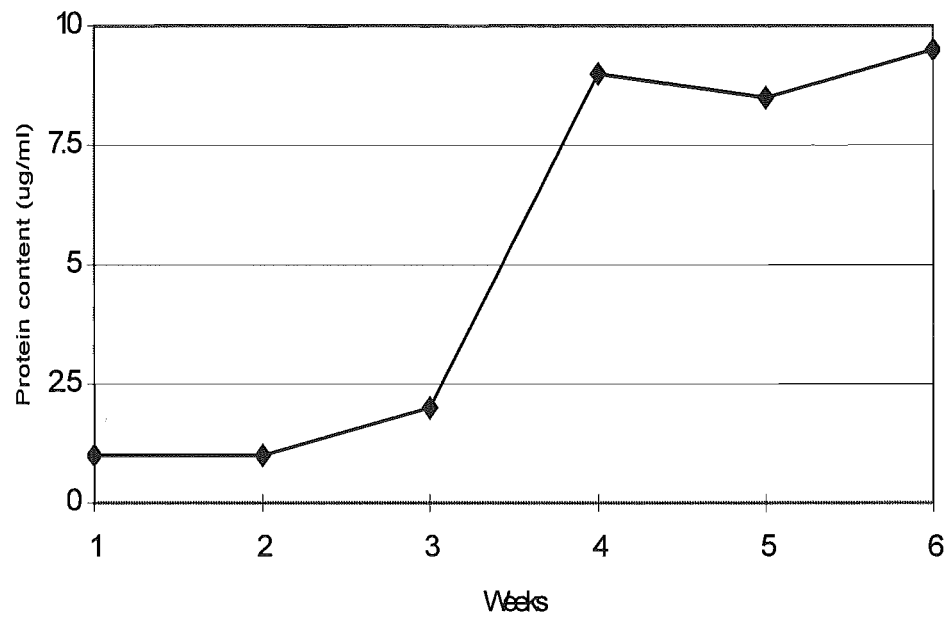


Fig. 59. Time course of soluble protein changes in the standard plantlet multiplication medium ('Iwa')

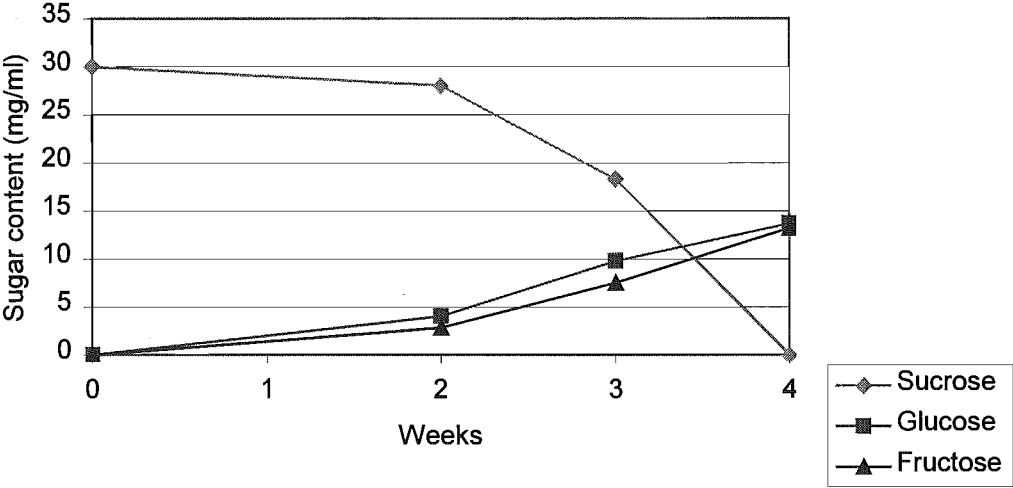


Fig. 60. Time course of carbohydrate changes in the standard plantlet multiplication medium ('Iwa')

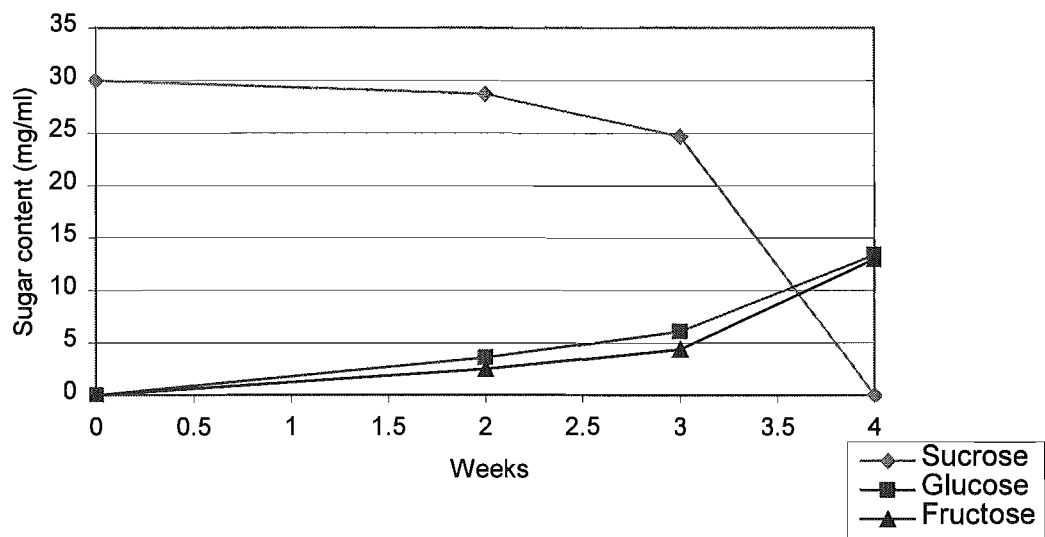


Fig. 61. Time course of carbohydrate changes in the standard plantlet multiplication medium ('Daeji')

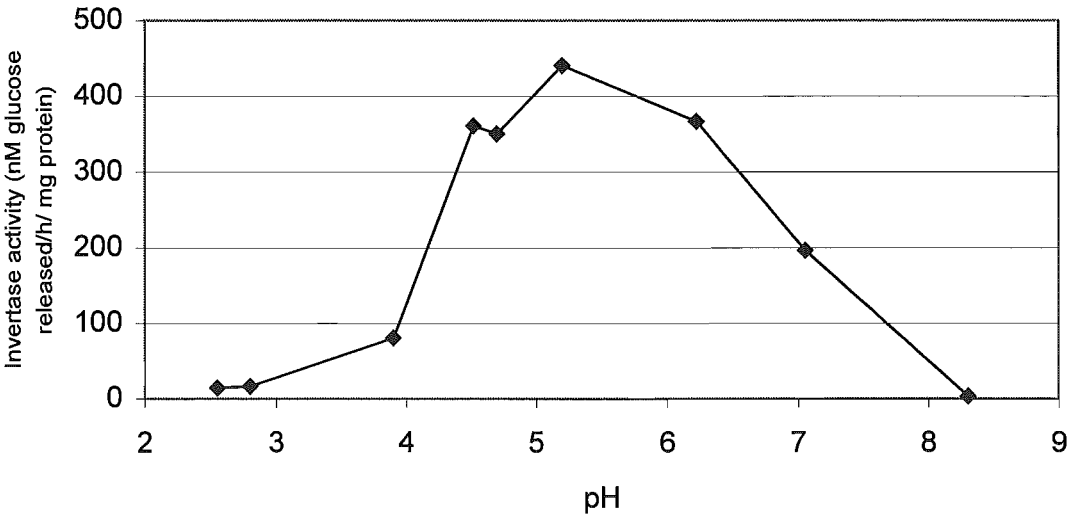


Fig. 62. pH profile of invertase activity in the plantlet multiplication medium ('Iwa').

activity in the multiplication medium was an acid invertase.

The activity of invertase in the medium during growth of 'Iwa' plantlets increased sharply reaching a maximum level after 3-4 weeks in culture (Figs. 63 and 64). Subsequently beyond the typical plantlet multiplication step the activity seemed to decrease.

2.1.2.4 Change in the pH of the multiplication medium

While the pH of the medium was initially adjusted to pH 5.8 which is close to the pH optimum for invertase activity in the medium, it was of interest to see if this remained so throughout the culture period during development of 'Iwa' and 'Daeji' plantlets and invertase development in the medium. The pH of the multiplication medium was measured every week for 4-5 weeks of culture. There was a gradual decline of pH during the first 3 weeks of culture. After 3 weeks, the pH increased slightly (Figs. 65 and 66).

2.1.2.5 Osmotic potential

Osmotic potential of the medium rose during development of 'Iwa' plantlets (Fig. 67) until week 4 before dropping rapidly when the multiplication stage was prolonged beyond the typical experimental period up to 6 weeks. The pattern of osmotic potential changes in the medium during development of 'Daeji' plantlets appears to be different (Fig. 68). There was an initial increase in osmotic potential which then remained the same throughout plantlet development before starting to decrease a week after the end of the multiplication stage.

2.2 Effects of variations to the standard multiplication medium

2.2.1 Different carbohydrate media for plantlet multiplication

2.2.1.1 Comparison of plantlet weights

When the standard multiplication medium was modified to contain no sugar or 3% (w/v) maltose instead of sucrose, it was found that sucrose was not required for the 2-nodal potato shoot explants of both varieties under continuous illumination in a stationary liquid medium. The fresh weight of plantlets increased markedly within 3 weeks of culture (Figs. 69 and 70).

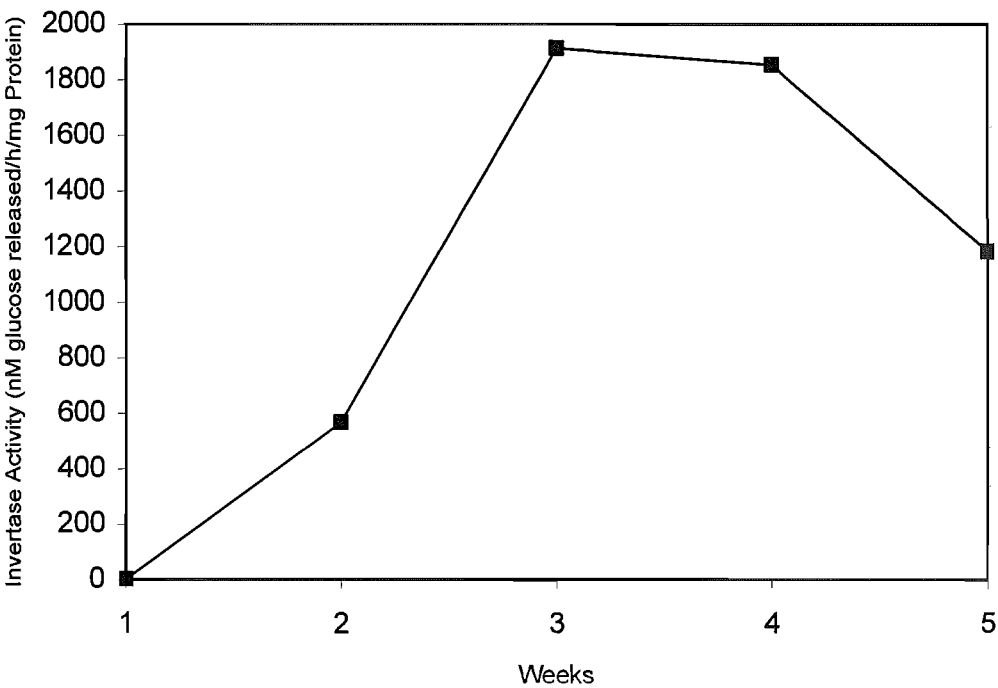


Fig. 63. Time course of invertase activity in the standard plantlet multiplication medium ('Iwa')

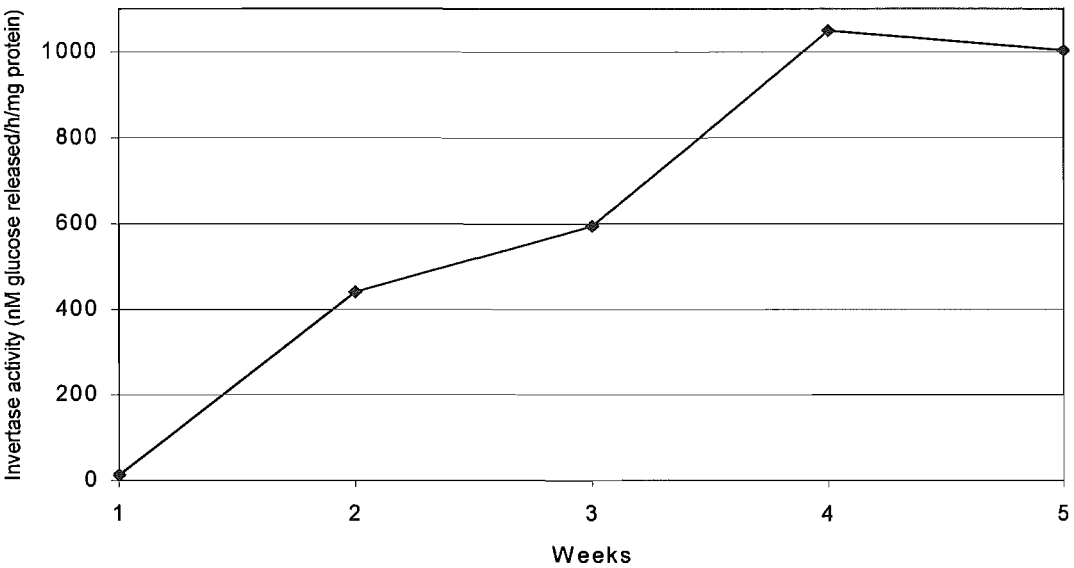


Fig. 64. Time course of invertase activity in the standard plantlet multiplication medium ('Daeji')

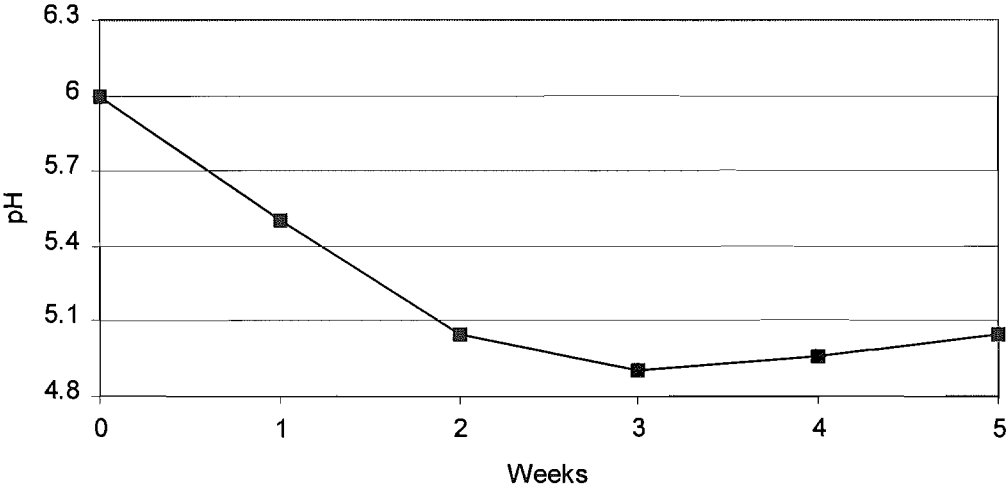


Fig. 65. Time course of pH changes in the standard plantlet multiplication medium ('Iwa').

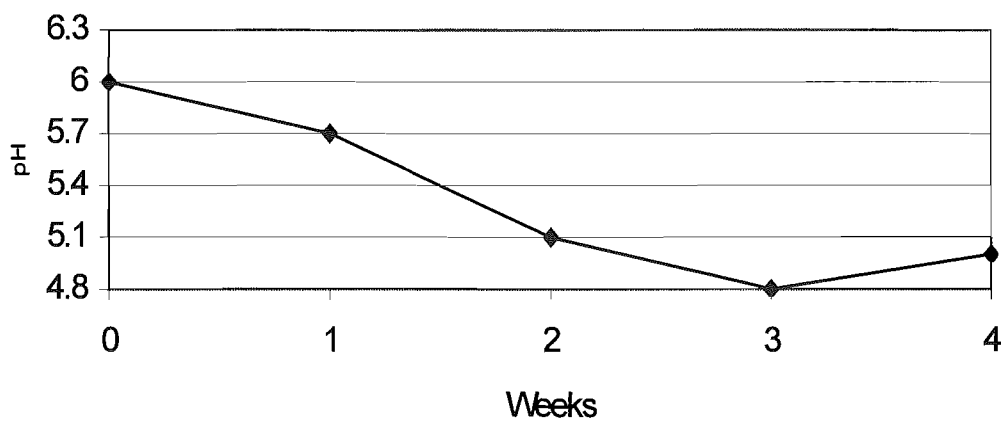


Fig. 66. Time course of pH changes in the standard plantlet multiplication medium ('Daeji').

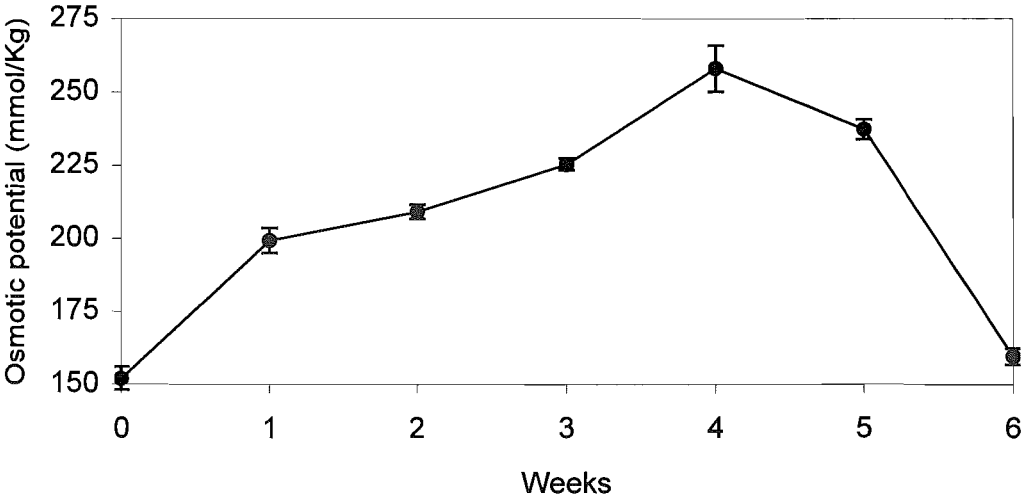


Fig. 67. Time course of osmotic potential changes in the standard plantlet multiplication medium ('Iwa').

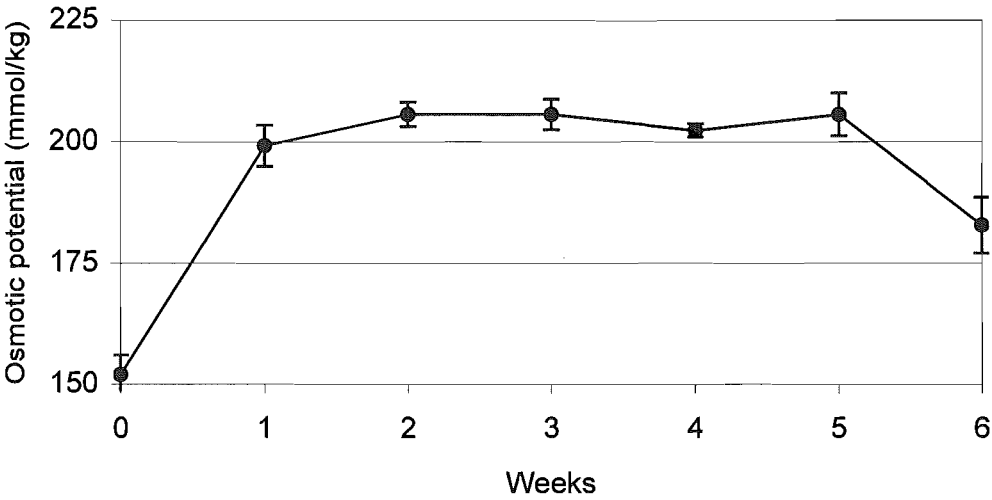


Fig. 68. Time course of osmotic potential changes in the standard plantlet multiplication medium ('Daeji').

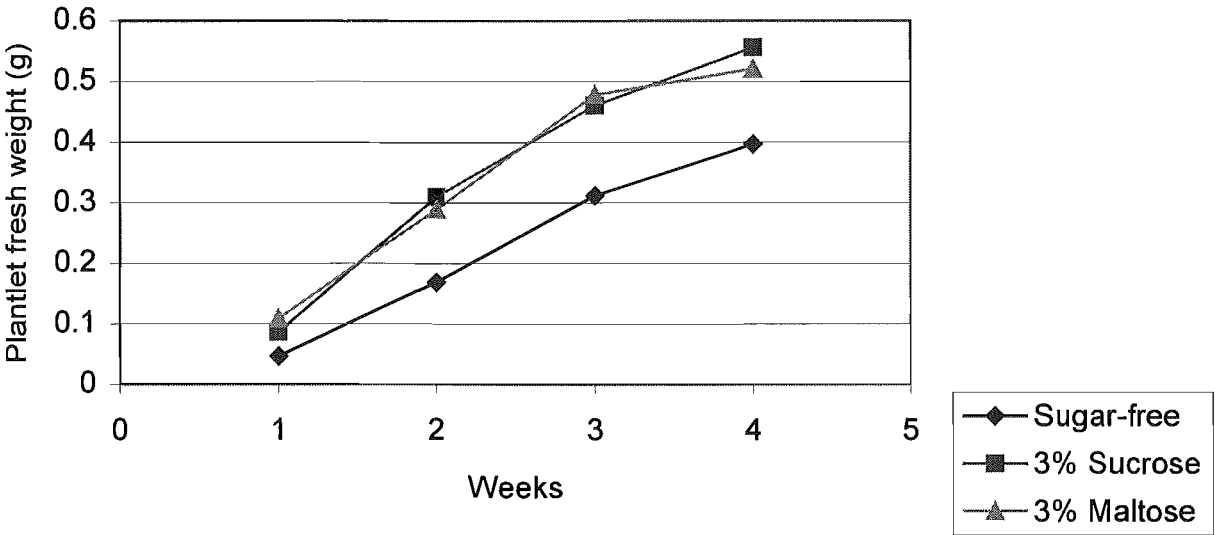


Fig. 69. Time course of plantlet growth in response to media containing different carbohydrates ('Iwa').

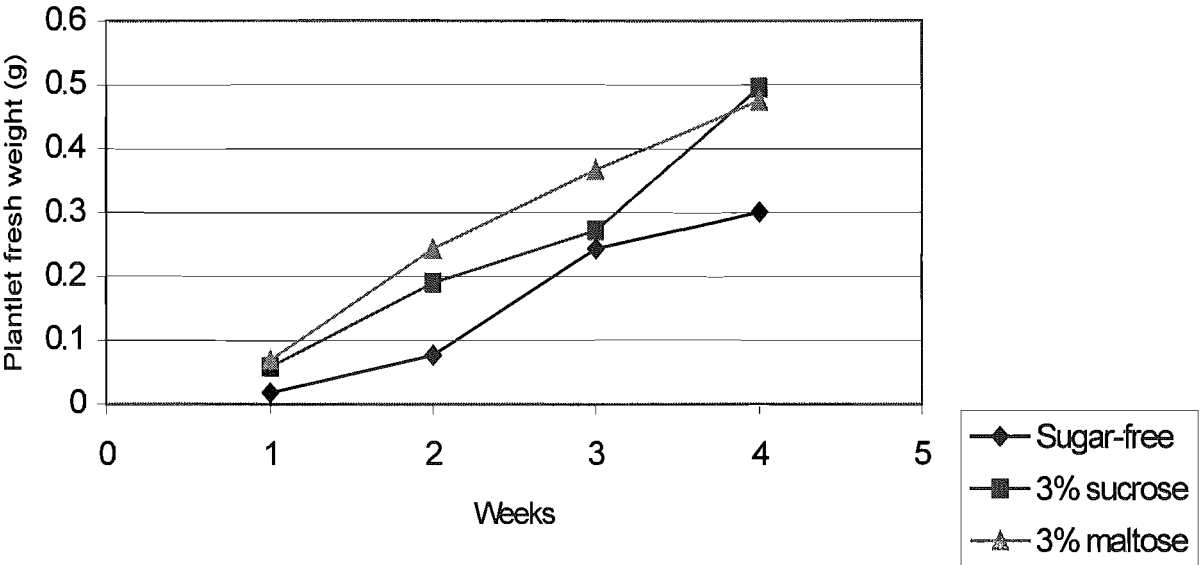


Fig. 70. Time course of plantlet growth in response to media containing different carbohydrates ('Daeji').

The plantlets in sucrose and maltose media had thicker and stronger stems compared to the weak appearances of the plantlets developed in sugar-free medium.

2.2.1.2 Time course of soluble protein changes in different carbohydrate media ('Iwa')

Soluble protein contents of all 3 media increased during plantlet development (Fig. 71). By week 4 of culture, there was little difference in the protein contents of the 3% sucrose- and 3% maltose-containing media. However, the sugar-free medium appeared to have lower levels of soluble proteins.

2.2.1.3 Time course of carbohydrate changes ('Iwa')

No sugar was detected in the modified multiplication medium without any added sucrose during plantlet multiplication. Maltose was apparently retained up to 4 weeks of culture in the medium supplemented with 3% (w/v) maltose in which little or a negligible amount of glucose was sometimes detected (Fig. 72).

2.2.2 Effect of replacing medium during multiplication on growth of plantlets

At the end of the multiplication step (i.e. after 4 weeks of culture), the plantlets grown in the medium that was replaced at the end of week 2 had developed thicker and stronger stems than those from the standard multiplication protocol. The fresh weights of the 'Iwa' and 'Daeji' plantlets from the medium that was replaced were also substantially greater than those in the standard multiplication protocol (Figs. 73 and 74). However, there was little difference in the dry weights of the plantlets between the two treatments at this point.

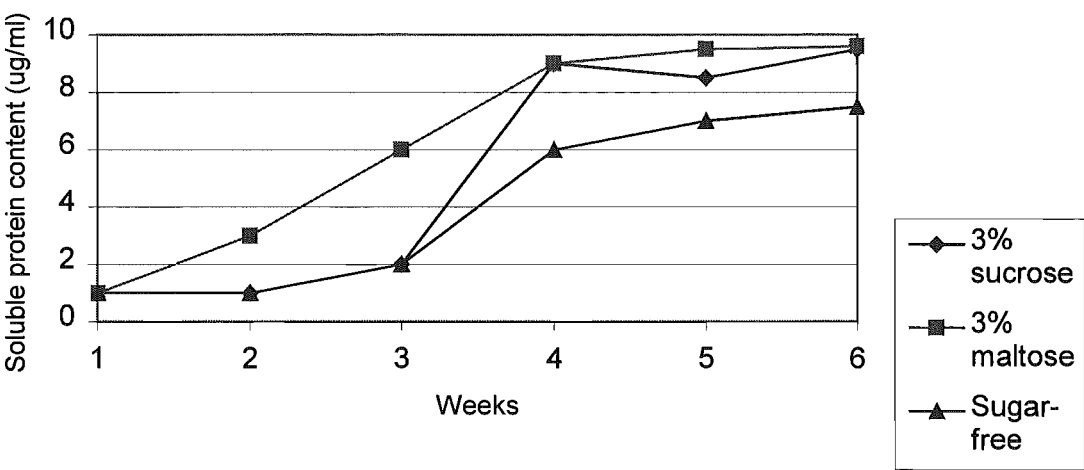


Fig. 71. Time course of soluble protein changes in containing different carbohydrates multiplication media ('Iwa').

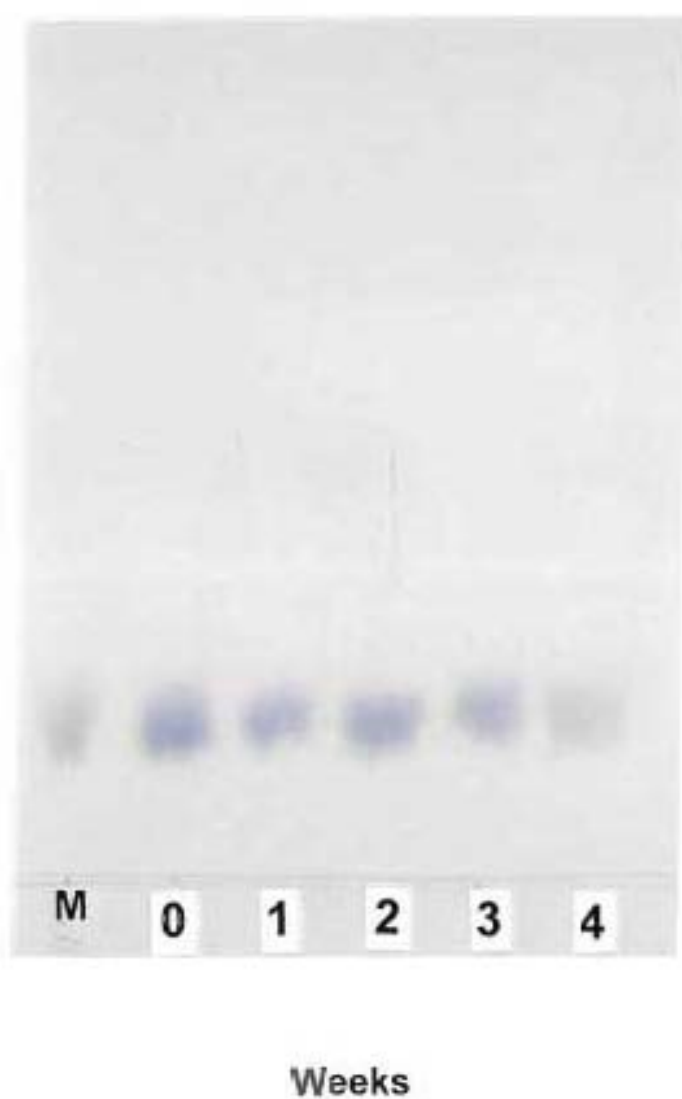


Fig. 72. Paper chromatography on time course of carbohydrate changes in the standard multiplication medium with maltose instead of sucrose ('Iwa'). Sugar standards: M=maltose.

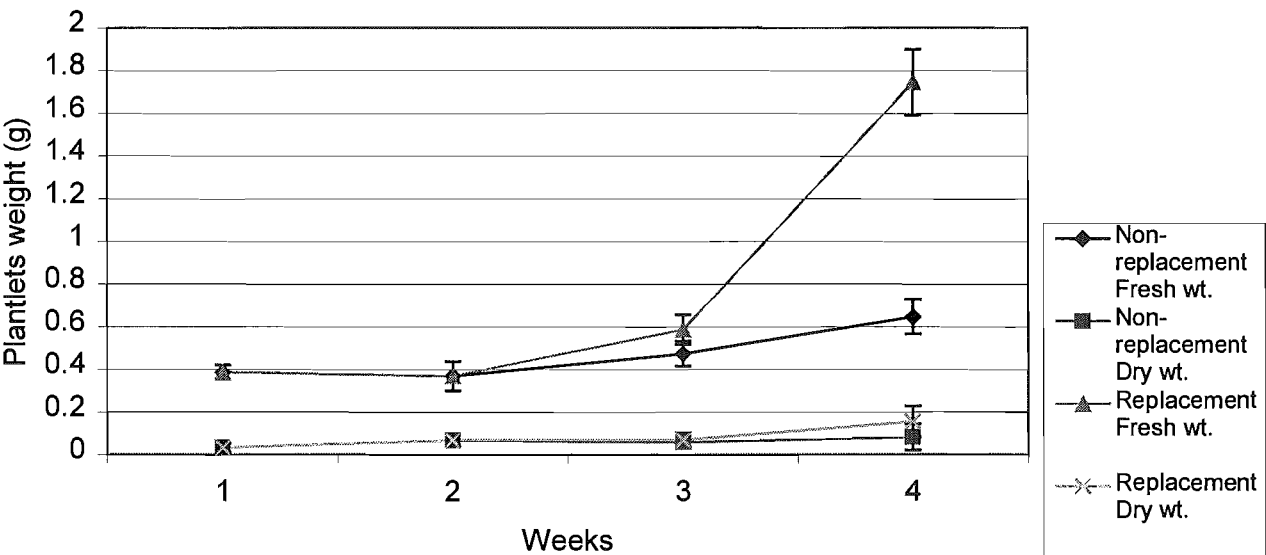


Fig. 73. Effect of medium replacement treatment on plant development ('Iwa').

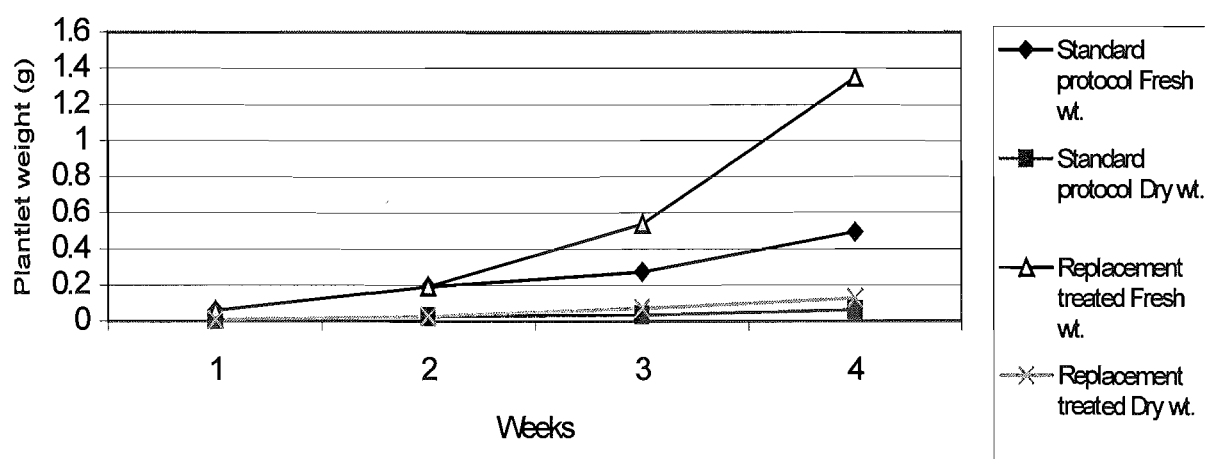


Fig. 74. Effect of medium replacement treatment on plant development ('Daeji').

2.2.3 Effect of manipulations during multiplication phase on microtuberization

2.2.3.1 Influence of carbohydrates in multiplication media on microtuberization under standard conditions

The effects of prior culturing of the plantlets in multiplication media containing different carbohydrates on the number of microtubers formed, their average fresh weights and relative size distribution following 10 weeks of the standard microtuberization protocol were investigated.

Visual examination suggested that the thicker and more vigorous shoots transferred from the multiplication media containing carbohydrates seemed to form more microtubers which were bigger than those formed by the thin and weak shoots transferred from sugar-free multiplication medium. The number of microtubers formed by 'Iwa' or 'Daeji' plantlets was found to be similar (about 3 per 3 plantlets) in the standard 8% sucrose-containing microtuberization medium regardless of whether the plantlets were transferred from the carbohydrate-free or the 3% maltose, or the standard 3% sucrose-containing multiplication media (Table 7).

The standard protocols, i.e. the transfer of 'Iwa' and plantlets from the standard multiplication medium containing 3% sucrose to the standard microtuberization medium containing 8% sucrose led to the highest average microtuber weight when compared to the transfer from the modified multiplication medium containing no sugar or 3% maltose to the standard microtuberization medium (Fig. 75). It is also interesting to note that these latter two protocols had virtually indistinguishable effects as far as average microtuber size is concerned. In the case of the same experiments with 'Daeji', the statistical analysis revealed no significant differences among the 3 treatments (Fig. 76; $p \leq 0.05$).

From an examination of the frequencies of microtubers in different weight categories (Fig. 77 and 78), it is clear that both the 'Iwa' and 'Daeji' plantlets transferred from the multiplication medium containing 3% sucrose, the concentration used in the standard multiplication medium, tended to form larger microtubers and fewer of the smaller ones when compared to the other two protocols (Figs. 77 and 78).

Table 7. Effect of different plantlet multiplication media on the number of microtubers formed in 8% sucrose tuberization medium.

Number of microtubers formed per 3 plantlets per jar

Multiplication medium treatments	‘Iwa’	‘Daeji’
Sucrose-free	2.33 ± 1.06	2.20 ± 1.06
Maltose	3.16 ± 1.56	2.80 ± 0.62
Sucrose	2.70 ± 0.23	3.20 ± 0.62

No significant difference was found among the treatments (ANOVA, $p \leq 0.05$)

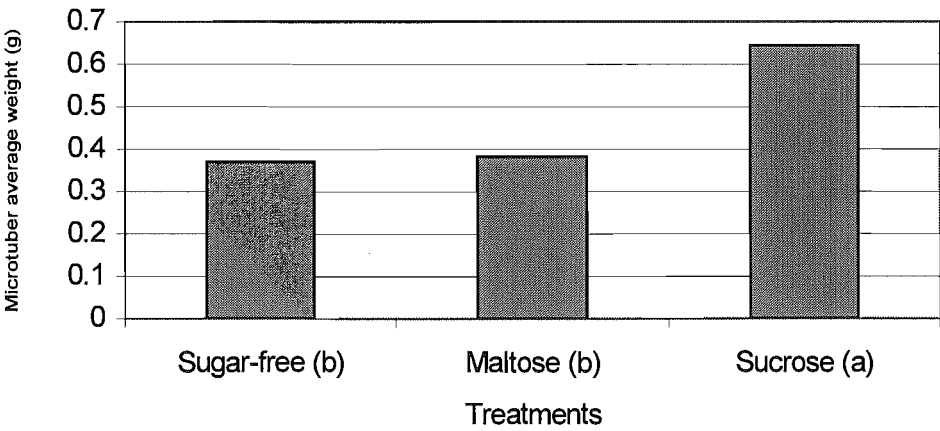


Fig. 75. Effect of different plantlet multiplication media on average fresh weights of microtubers harvested at the end of the standard microtuberization step ('Iwa')
Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.01 level.

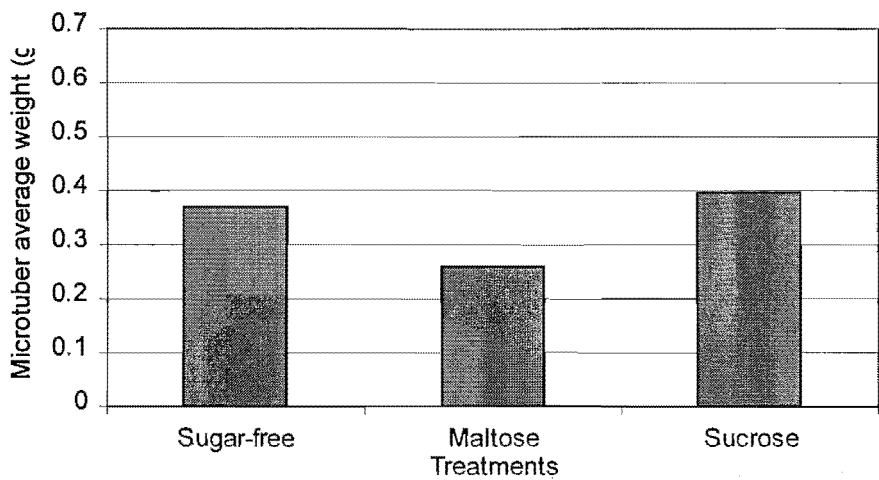


Fig. 76. Effect of different plantlet multiplication media on average fresh weights of microtubers harvested at the end of the standard microtuberization step ('Daeji'). No significant difference was found among the treatments (ANOVA, $p \leq 0.05$)

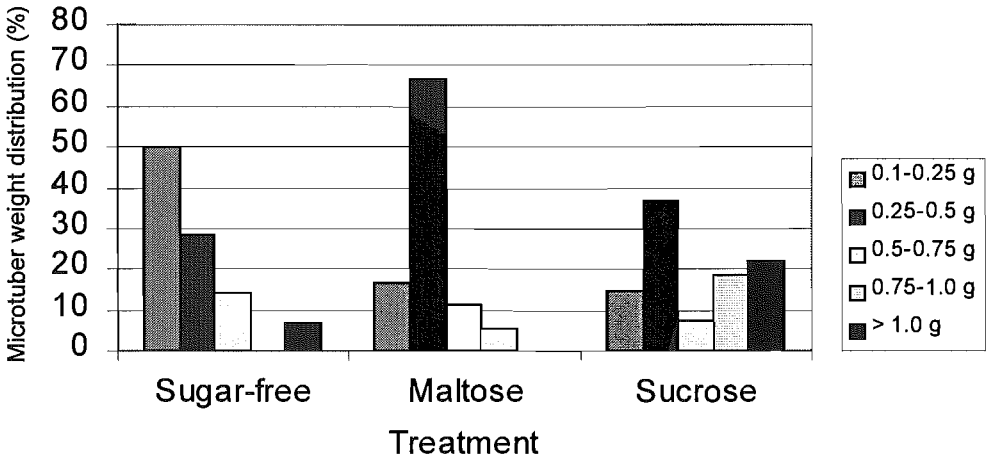


Fig. 77. Effect of different multiplication media on fresh weight distribution of microtubers harvested at the end of the standard microtuberization step ('Iwa').

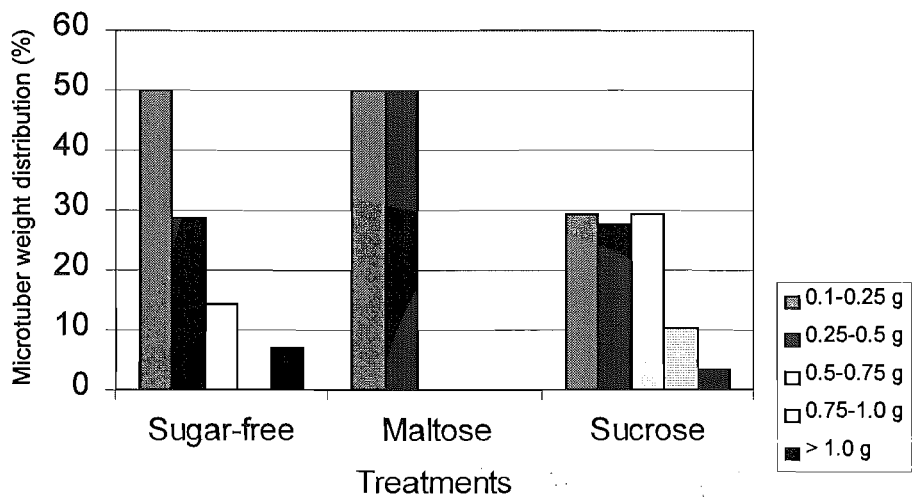


Fig. 78. Effect of different plantlet multiplication media on fresh weight distribution of microtubers harvested at the end of the standard microtuberization step ('Daeji').

2.2.3.2. Effect of replacing medium during multiplication on microtuberization

Following 10 weeks of the standard microtuberization protocol, the effects of prior culturing the plantlets on multiplication media that were replaced during potato plantlet multiplication on the number of microtubers formed, their average fresh weights, and relative size distribution, were investigated.

In both 'Iwa' and 'Daeji' cultivars, replacing the multiplication medium did not seem to have a substantial effect on the number of tubers formed in the subsequent standard tuberization protocol (Table 8). The thicker and more vigorous shoots resulting from replacing the multiplication medium of 'Iwa' and 'Daeji' plantlets, led to the subsequent formation of larger microtubers (Fig. 79) than found on the plantlets grown in the standard multiplication multiplication protocol (i.e. the medium was not changed).

An examination of the frequencies of microtubers formed by 'Iwa' or 'Daeji' plantlets in different weight categories revealed that replacing the medium during the standard multiplication step led to the production of some microtubers heavier than 2.0g (Figs. 80 and 81). This category was rarely observed in other treatments including the standard multiplication and microtuberization protocols.

Table 8. Effect of medium replacement during multiplication on the number of microtubers formed at the end of the standard microtuberization step.

Medium treaments	‘Iwa’	‘Daeji’
Replaced	3.20 ± 1.31	3.23 ± 1.35
Standard protocol	2.71 ± 0.23	2.90 ± 0.76

No significant difference was found among the treatments (ANOVA, $p \leq 0.05$)

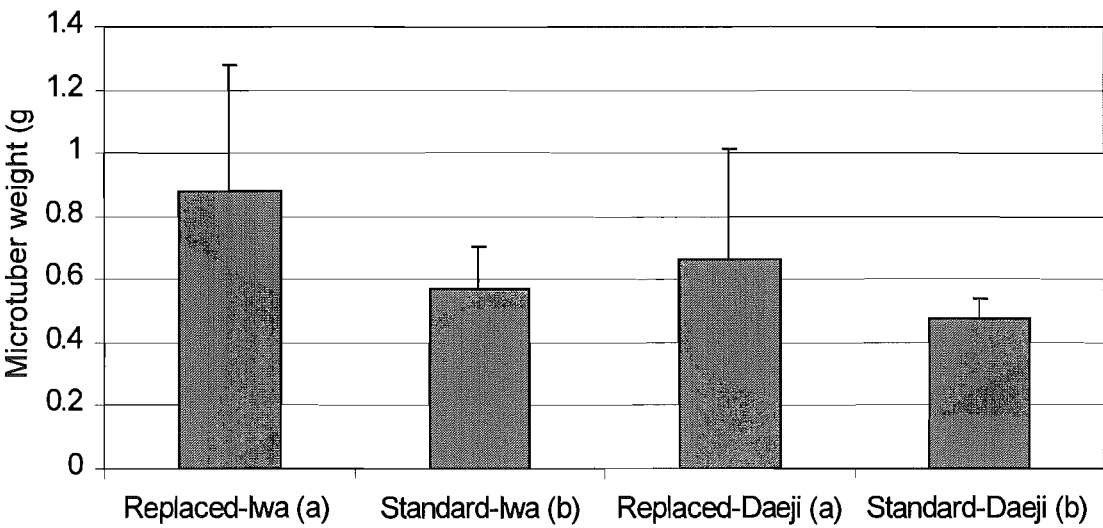


Fig. 79. Effect of medium replacement during multiplication on average fresh weights of microtubers harvested at the end of the standard microtuberization step. Treatments with different letters are significantly different according to Bonferoni's comparison of means at the 0.05 level.

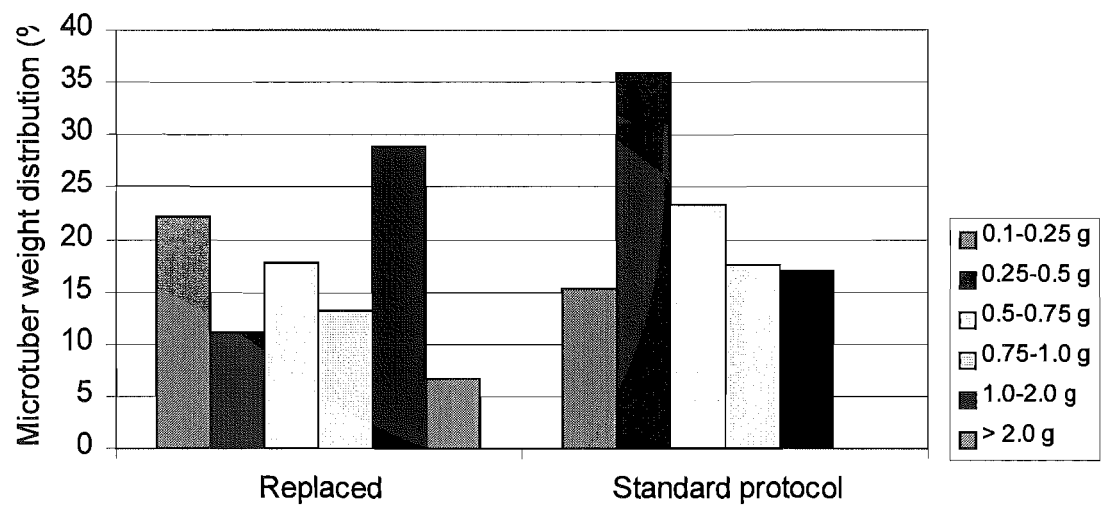


Fig. 80. Effect of medium replacement during multiplication on fresh weight distribution of microtubers harvested at the end of the standard microtuberization step ('Iwa'). Standard protocol= no medium replacement.

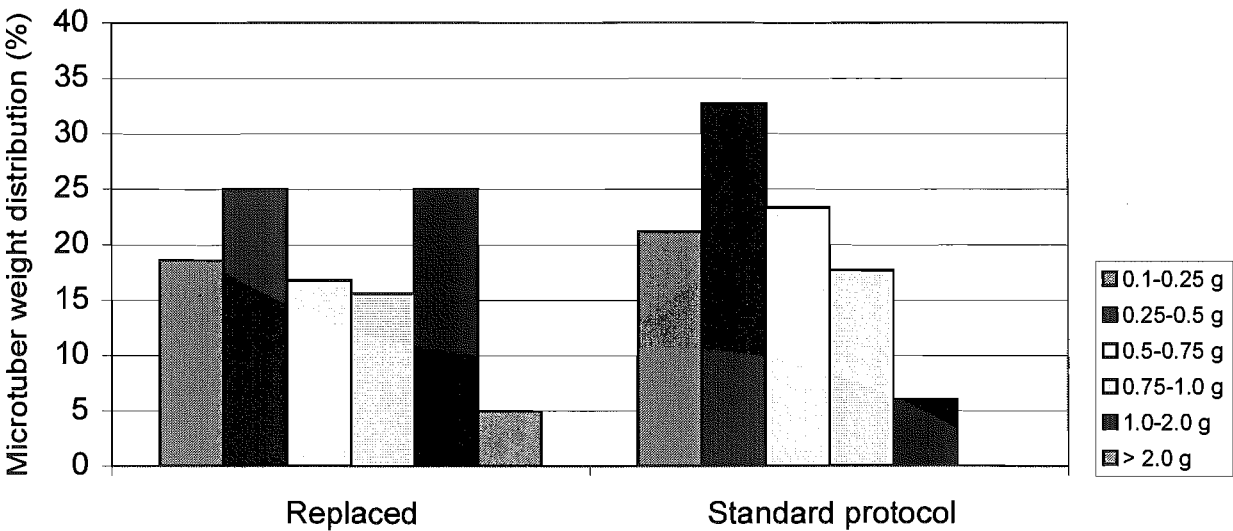


Fig. 81. Effect of medium replacement during plantlet multiplication on fresh weight distribution of microtubers harvested at the end of the standard microtuberization step ('Daeji').

3. Ultrastructural Observations

In the course of this study, several observations seem to deserve more in-depth physiological and biochemical investigations. First, some parts of the stem and leaves of plantlet were submerged in the liquid multiplication and microtuberization medium. Second, at the end of the plantlet multiplication step, the roots turned green and some remained so in the subsequent microtuberization step. Third, some white clusters of cells became floating freely in the medium during the microtuberization step. The implications of these observations for the efficiency of plantlet growth or microtuberization are unknown and could be significant. In the present study, it was decided to conduct an ultrastructural survey with particular emphasis on the features of chloroplasts or plastids in the various tissues of 'Iwa'.

3.1 Stem and leaf of *in vitro* potato plantlets grown in liquid medium

The upright, vigorous shoot tissues of plantlets that were above the liquid medium surface at the end of the standard multiplication step had well-developed chloroplasts with typical ultrastructural features (Plates 1 and 2). The granal stacks were relatively small and the grana formed irregular continuous stacks, interconnected by stroma thylakoids. Within the stroma plastoglobuli were found (Plate 1). In the stem, granal stacks of the chloroplasts displayed a few large continuous stacks mostly regular in shape, occupying most of the organelle (Plate 2). Plastoglobuli were also found. In the chloroplasts of both leaf and stem, no starch grains were found.

3.2 Stem and leaf cells submerged in liquid medium at the end of the standard multiplication step

Some chloroplasts in green stems that had been submerged in liquid medium throughout the multiplication step possessed starch grains (Plate 3). The matrix of chloroplasts contained plastoglobuli. Microbodies

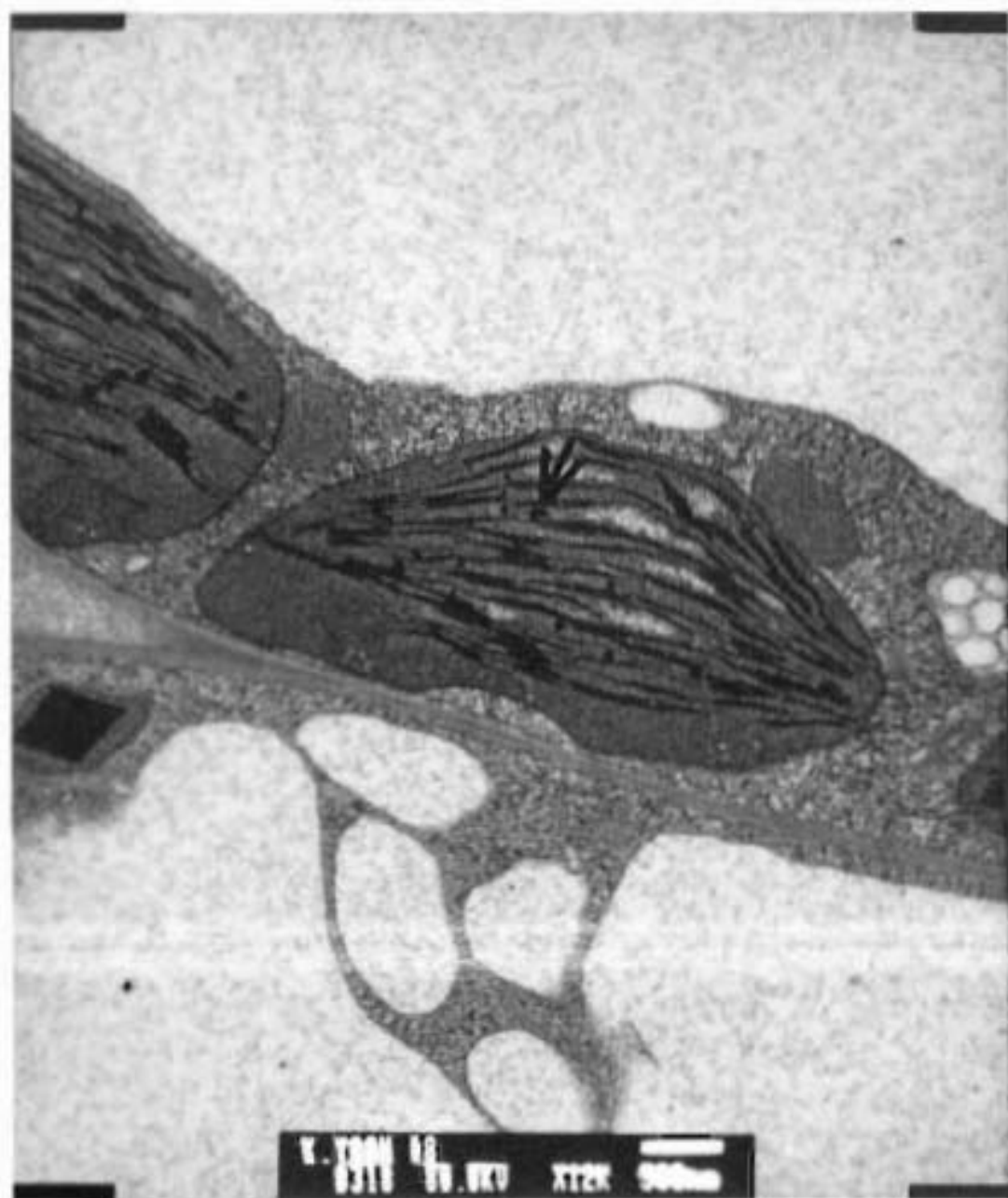


Plate 1. TEM view of chloroplast with small plastoglobulus (arrowhead) in leaf of *in vitro* potato plantlet ('Iwa) grown in liquid medium. Magnification= (12,000x)



Plate 2. TEM view of mesophyll chloroplast in stem of *in vitro* potato plantlet ('Iwa') grown in liquid medium with thylakoid grana and a small plastoglobuli (arrowhead). Magnification—(15,000x)



Plate 3. Typical chloroplast organization in the part of the green stem that was submerged in the liquid plantlet multiplication medium. The granal stacks were relatively small, mostly irregular in shape and interconnected by stroma thylakoids. Within the stroma the plastoglobuli were scattered. Magnification = (15,000x)

Microbodies were also observed alongside chloroplasts (Plate 4). Chloroplasts were also found in the parenchyma of leaves that were submerged in liquid medium, but ultrastructural features appeared to be in a more compressed state. In these chloroplasts, the granal stacks were mostly parallel to the long axis of the organelle (Plate 5). They represented a type of chloroplast that contained less developed thylakoids and plastoglobuli (Plate 6) than those found in the non-submerged stem.

3.3.1 Stem and leaf after microtuberization in liquid medium in the dark

Amyloplasts with large starch grains were dominant in stem or leaf submerged in liquid medium at the end of the standard microtuberization step. The amyloplast had an irregular appearance (Plates 7a and 7b).

3.3.2 Green roots in liquid tuberization medium

At the end of 10 weeks of the standard microtuberization step, some roots that remained green and some were white. Both types of roots were harvested for TEM study. Green roots maintained their typical root anatomy. The cortical cells contained abundant chloroplasts, mostly plano-convex in shape, located at the periphery of the cell (Plates 8a and 8b). These showed typical cortical cell ultrastructure. Their chloroplasts had well-differentiated thylakoids, plastoglobuli and starch grains. Granal stacks were more abundant, while some had a complex and irregular shape. Overall the chloroplasts found in the green roots were very similar to the chloroplasts found in leaves.

In contrast, the plastids of cortical cells from non-greened roots remained largely undifferentiated and in some cases had starch granules. Within the stroma matrix of the plastid ovoid starch grains were often surrounded by an electron transparent zone (Plate 9). Dictyosomes were typical of storage parenchyma cells from active and young tissues, and showed vesicles of varied sizes (Plate 10).

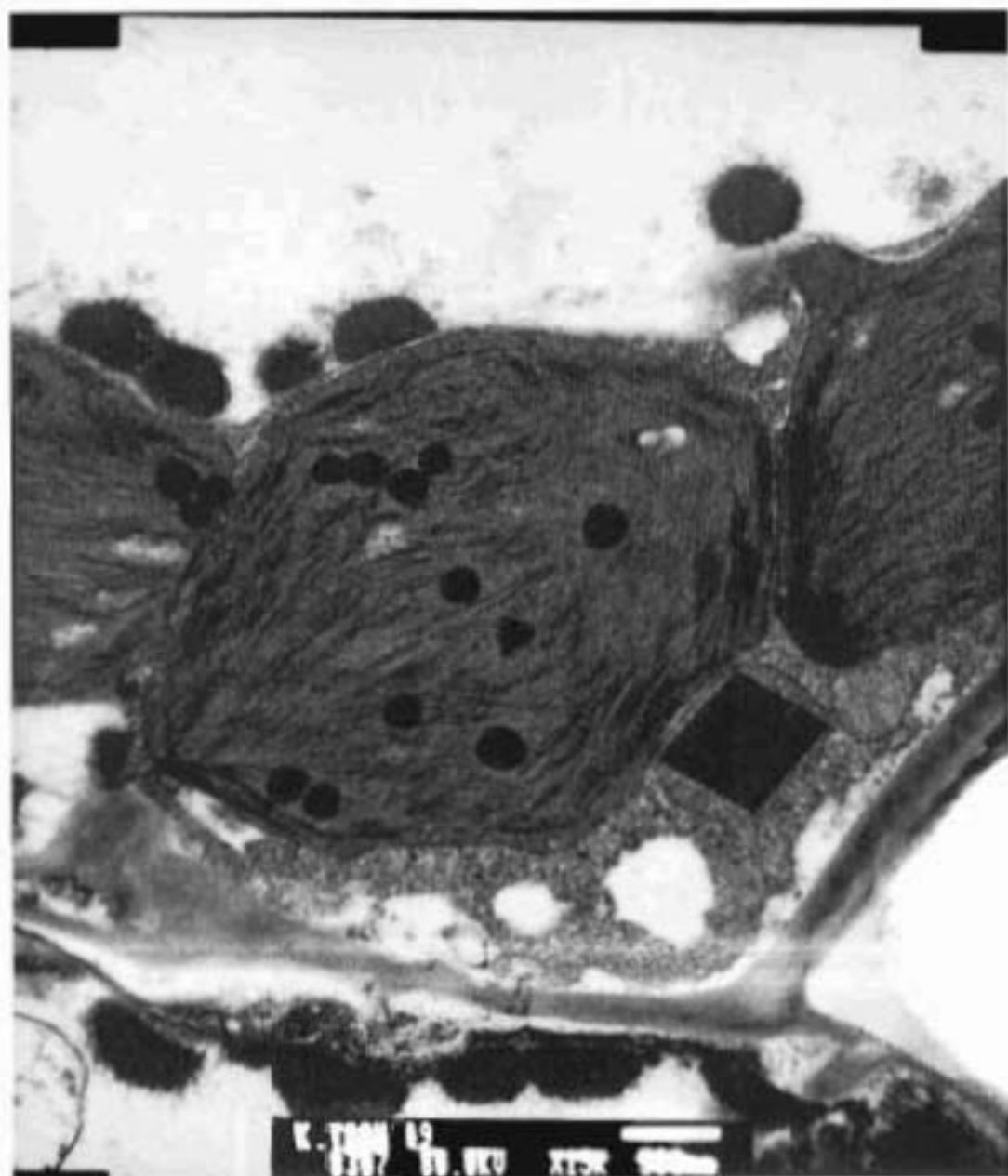


Plate 4. Grana and thylakoids in the stem tissue that was submerged in liquid medium at the end of the standard multiplication step. Granal stacks are abundant. Microbody alongside chloroplasts. Magnification=(15,000x)



Plate 5. TEM view of mesophyll chloroplast in leaf tissue that was submerged in liquid medium, fixed at the end of the standard multiplication step, with starch (s) and large plastoglobuli (arrowhead). The granal stacks were mostly continuous and composed of appressed thylakoids. Magnification \times (15,000s)

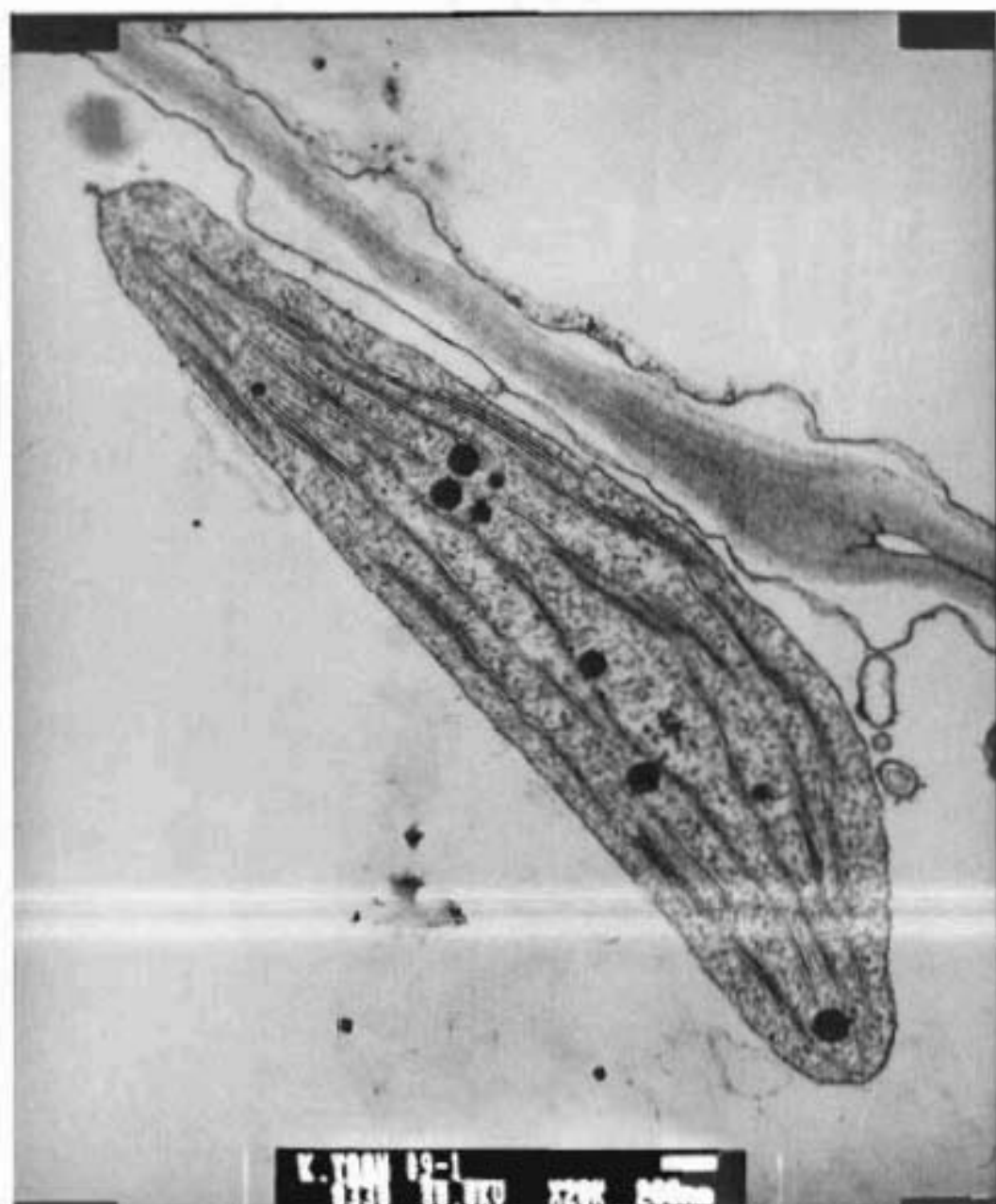


Plate 6. TEM view of chloroplast with small plastoglobuli in leaf tissue submerged in liquid medium at the end of the standard multiplication step. It had less developed thylakoids than that found in stem that was not submerged. Magnification $\times 20,000$

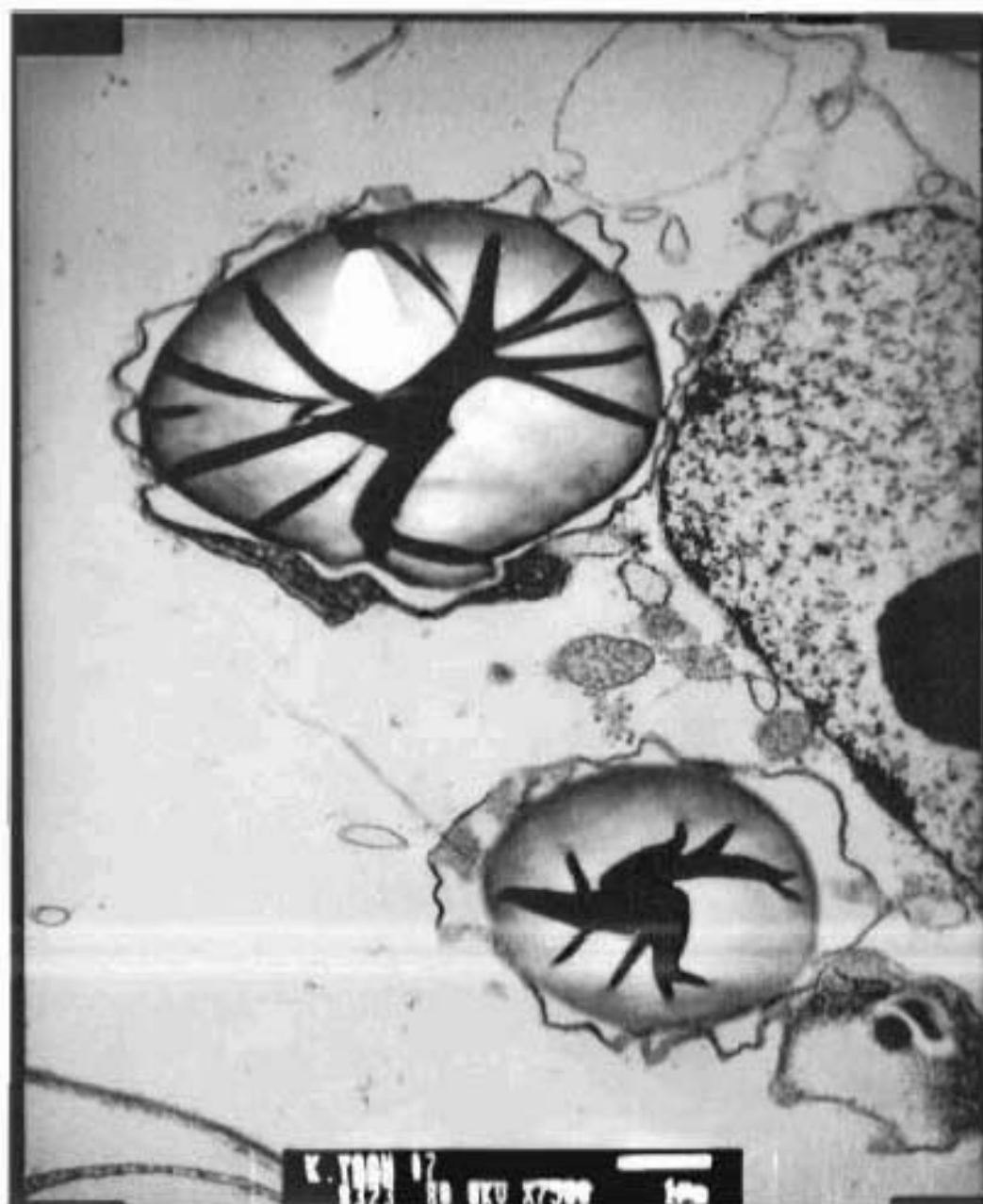


Plate 7a. Amyloplasts with large starch grains in stem tissue that was submerged after microtuberization step in liquid medium in the dark.
Magnification=(7,500x)



Plate 7b. Amyloplasts with large starch grains in leaf tissue that was submerged after microtuberization step in liquid medium in the dark.
Magnification: (15,000x)

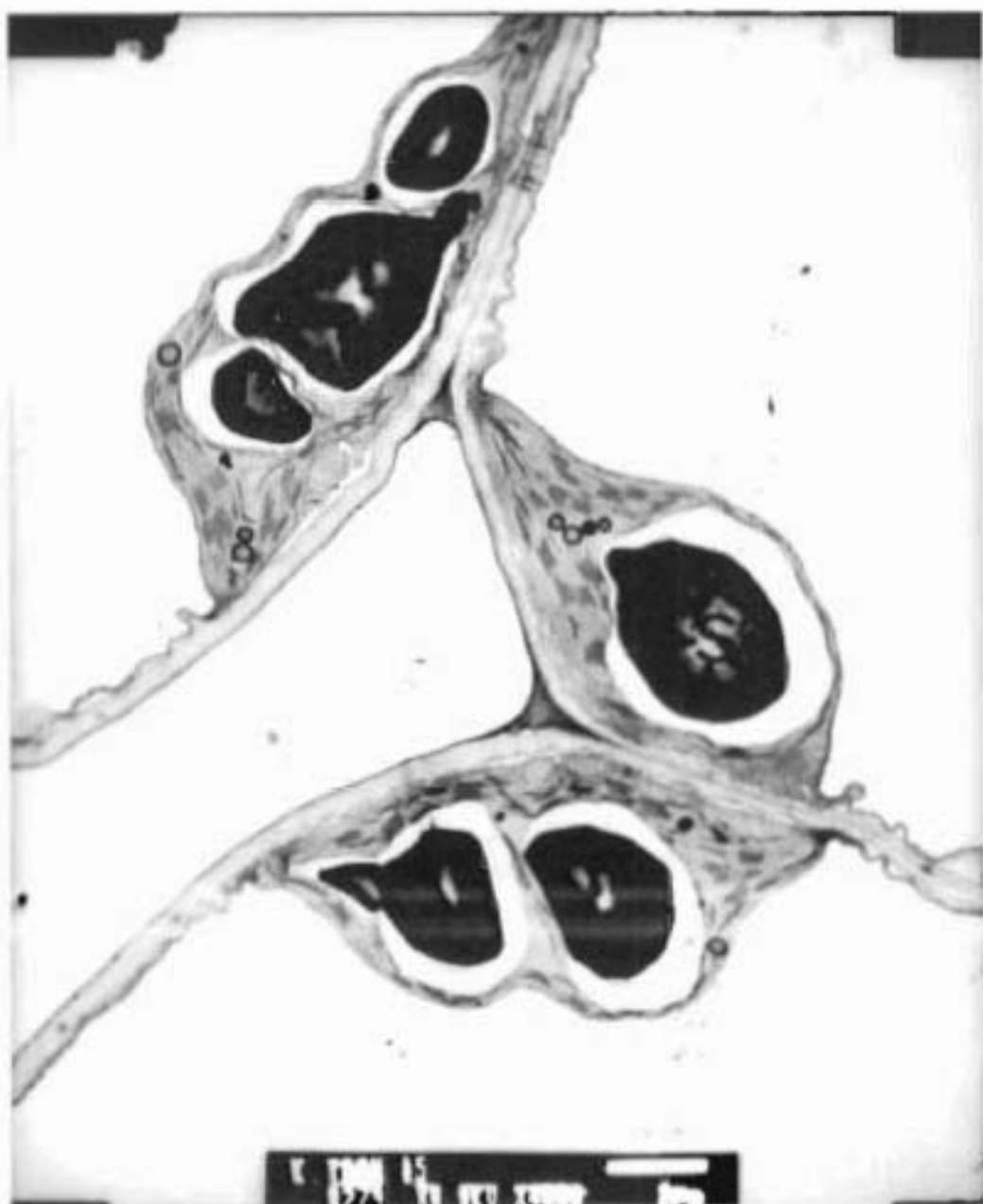


Plate 8a. Chloroplast in cortical cells of photoautotrophic green roots. Chloroplast with large starch grains (s) and a few small plastoglobuli. Magnification—(4,000x)



Plate 8b. Chloroplast in cortical cell of green roots. Chloroplast with large starch grains (s), surrounded by an electron transparent zone and a few small plastoglobuli. Magnification—(7,500x)



Plate 9. TEM view of a plastid of cortical cells from non-greened root in liquid tuberization medium. The plastid remained largely undifferentiated apart from starch granules. Magnification=(25,000x)



Plate 10. Typical TEM view of dictyosome of storage parenchyma cells with a few plastoglobuli from non-green roots in liquid tuberization medium.
Magnification-(25,000x)

3.3.3 Microtubers in liquid medium

In non-greened microtubers in liquid medium in the dark, the plastids of tuber cells remained largely undifferentiated and had large starch grains which suggests some organelles were destroyed by the long-term dark period. Microbodies had a granular matrix with a crystalloid center (Plates 11 and 12). Mitochondria (Plate 12) had cristae, and a matrix with a low electron density, which is characteristic of those from inactive or aged tissues.

In secondary microtubers, ovoid amyloplasts had a larger stromal volume with wide electron transparent zone and the lamellae were more or less parallel to each other (Plate 13). Mitochondria had a more electron dense matrix and more well-developed cristae suggesting that they were functionally active organelles. The space between lamellae was very narrow. Starch granules showed irregular shapes.

3.3.4 Floating cells in liquid tuberization medium

Oval amyloplasts were found in cells floating in liquid microtuberization medium. They contained large starch grains, which had irregular shapes (Plate 14). The amyloplast envelopes were less tortuous. Other organelles were also clearly evident. The origins of these non-green cells remained obscure.

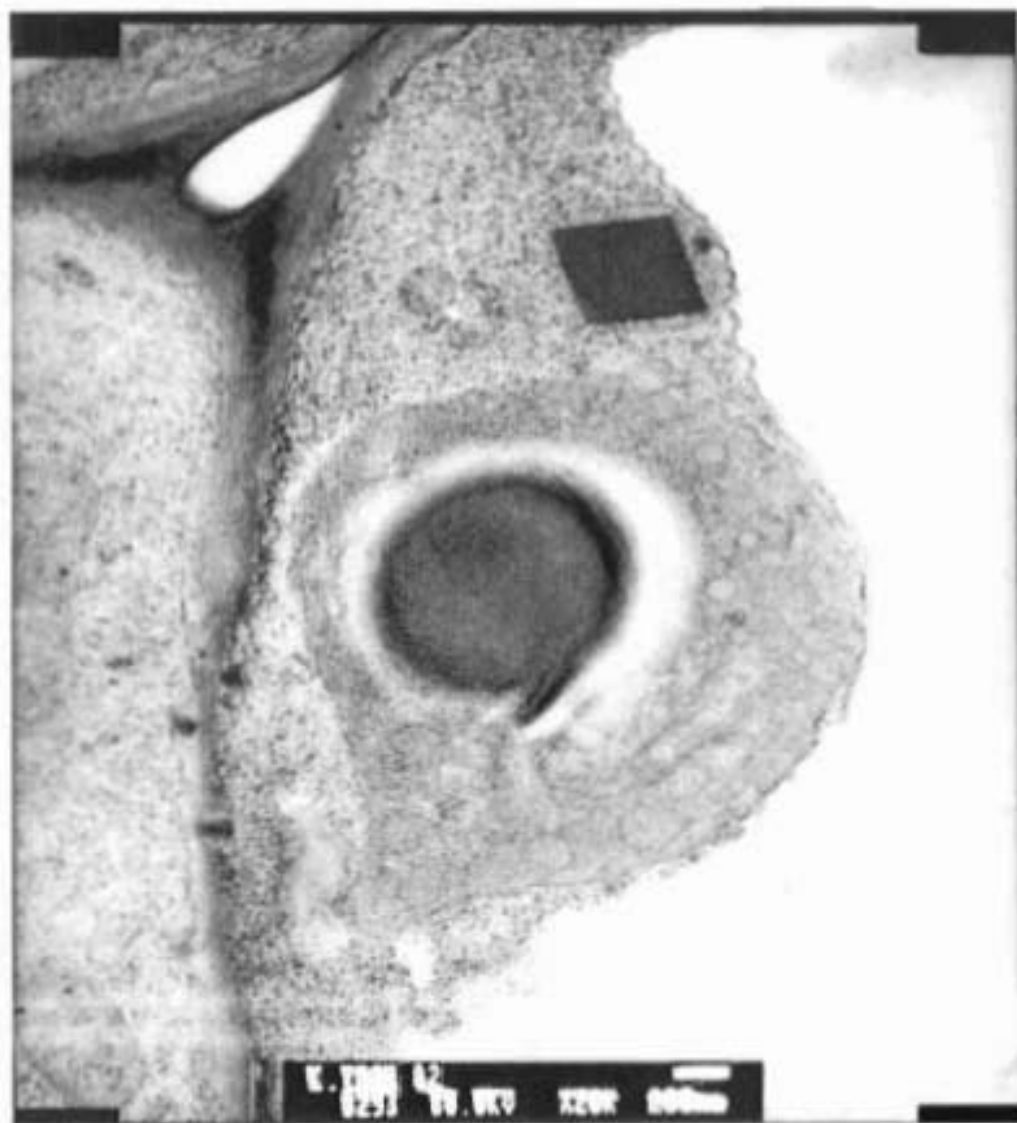


Plate 11. Plastid of tuber cells which remained largely undifferentiated with a large starch grain in a non-greened microtuber in liquid medium. Microbody had a granular matrix with crystalloid center. Magnification $\times 20,000$ s.



Plate 12. Mitochondria had cristae, and a matrix with a low electron density, in a non-green microtuber in liquid medium. Magnification $\sim (40,000\times)$

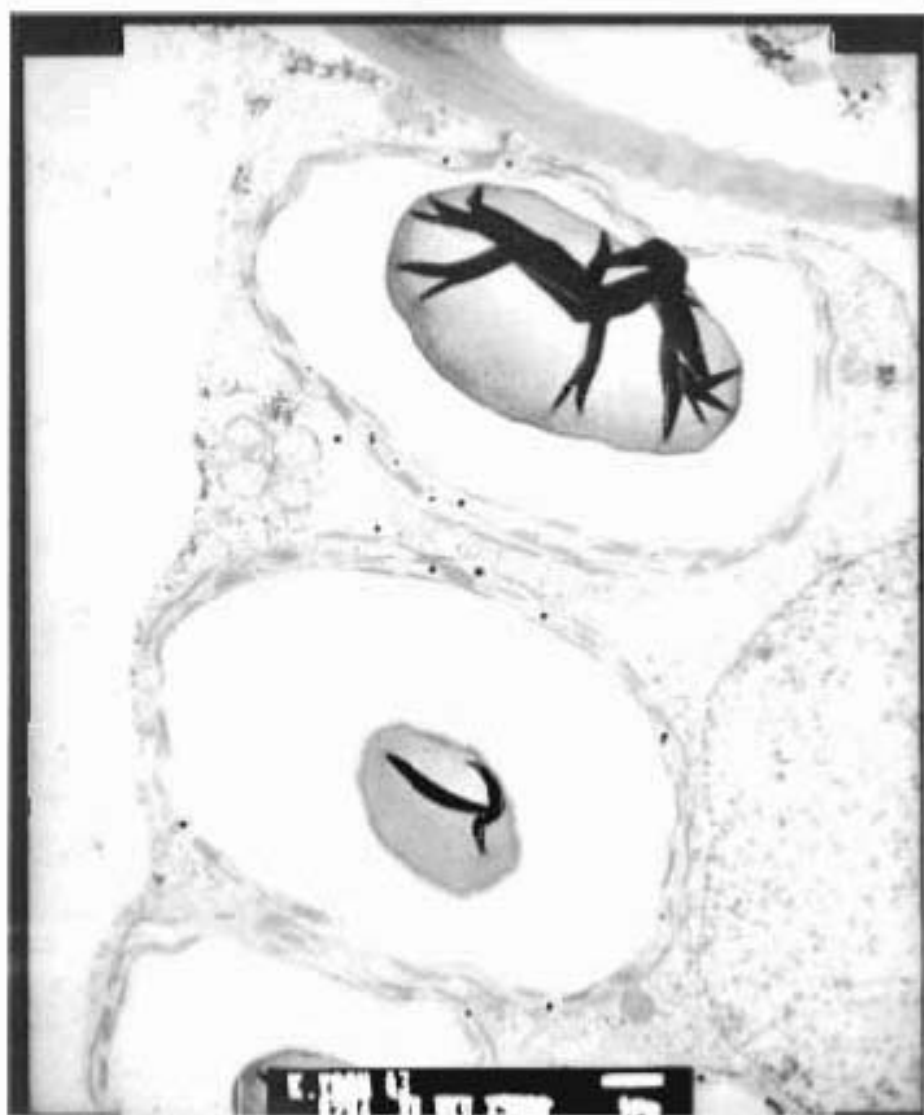


Plate 13. Ovoid amyloplast of a non-green microtuber harvested at the end of the microtuberization step had a larger stromal volume with wide electron transparent zone and lamellae were more or less parallel to each other. Magnification $\times(5,000\times)$

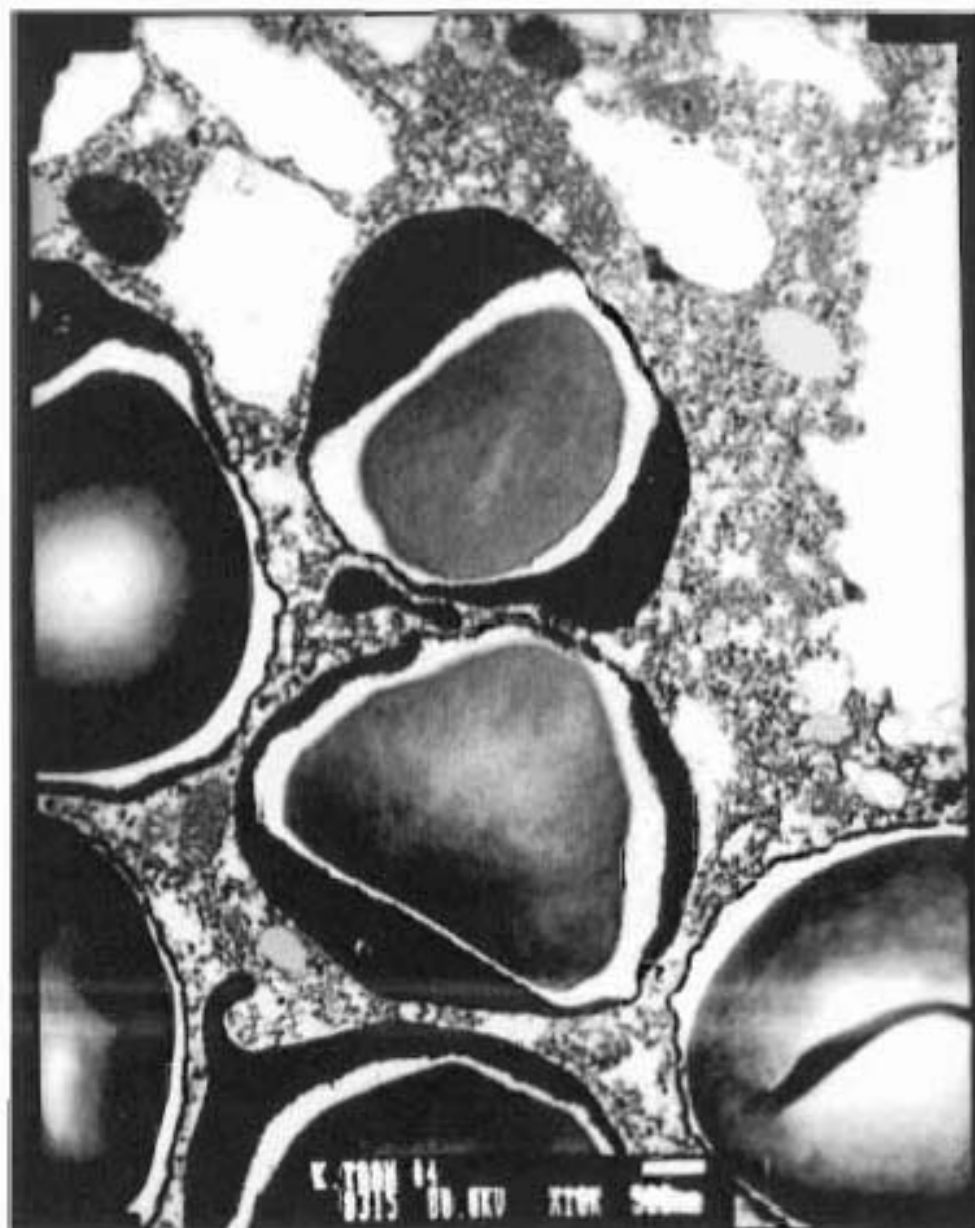


Plate 14. Oval amyloplasts in cells floating in liquid microtuberization medium contained irregular shaped large starch grains. The cells were harvested at the end of the microtuberization step. Magnification: (10,000x)

IV. Discussion

The advantages of studying the manipulations of media on the plantlet multiplication or microtuberization step separately become clear in the following discussion on the major findings of the present study.

1. Is sucrose superior to glucose or fructose for potato microtuberization?

There are studies comparing the effects of different carbohydrates on potato microtuberization although there is no clear explanation in the literature on the functions of carbohydrates in microtuberization medium compared at concentrations ranging from 4 to 12% (Chandra *et al.* 1988).

For example, it was observed that 8% sucrose was optimal for the development and growth of microtubers, while smaller microtubers were obtained on medium containing glucose or fructose, and none on that containing mannose or mannitol (Khuri & Moorby 1995). These authors also observed that fewer microtubers were produced on glucose than sucrose medium. Previous studies did not examine the microtuberization response of well-grown *in vitro* potato plantlets to different carbohydrates. In particular, it has not been established whether sucrose is superior to its constituent sugars, glucose and fructose.

Hence in this present study, the effects of glucose and fructose on potato microtuberization were studied singly or as a mixture of glucose and fructose and compared to sucrose in the standard tuberization medium (Fig. 19-24).

In addition, two possible criticisms of this type of study were addressed. To avoid any initial difference in osmotic potential or carbon content among the different media, the three monosaccharide-containing media were adjusted to be osmotically or carbon content equivalent to the standard tuberization medium containing 8% sucrose. The results on microtuber number and average microtuber weight did not show any clear advantage of the sucrose-containing medium over the other three monosaccharide-containing media. However, an examination of the microtuber size distribution revealed that while there was no difference between the sucrose- and the glucose + fructose-containing media, they were superior to the glucose-containing medium as they favored the formation of more larger microtubers than the latter medium. The effect of the fructose medium more resembled

that of the sucrose- or the glucose + fructose-containing medium. Taken together, our results seem to indicate that the invertase-mediated hydrolysis of sucrose in the standard tuberization medium is not essential for microtuberization.

2. Can another disaccharide replace sucrose in the standard tuberization medium?

Most potato (*Solanum tuberosum* L.) anther culture protocols recommend sucrose as the sole carbon source (Sopory 1979; Wenzel *et al.* 1983). However, some studies have suggested that sucrose may not be the ideal carbohydrate for all tissue culture systems (Ben-Hayyim & Neumann 1983; Fowler 1978; Keller *et al.* 1975; Kochba & Spiegel-Roy 1973). In particular, maltose (an α 1-4 linked glucose disaccharide) has been found to improve embryo induction and development in anther culture of petunia (Raquin 1983), somatic embryogenesis of alfalfa (*Medicago sativa*) (Strickland *et al.* 1987) and anther culture in potato (Batty and Dunwell 1989) and in a number of other species, particularly conifers.

In this study, similar tuberization results were obtained with maltose medium and the standard sucrose-containing tuberization medium (Table 4(a), 4(b); Fig. 37). Khuri & Moorby (1995) investigated microtuberization on media supplemented with 4% sucrose and one of the following sugars: sucrose, maltose, glucose or fructose, while adjusting the initial osmotic potential of the media to be equivalent to 8% sucrose. They found that significantly fewer microtubers formed on media with 4% sucrose supplemented with monosaccharides or maltose than with sucrose (equimolar media). However, their experiments were more complicated than ours for direct comparison. Particularly, there are two different aspects of experimental design. Firstly, in their study, the effect of a mixture of sucrose and maltose was studied, not maltose alone. Secondly, maltose was supplied in the media right from the beginning of plantlet multiplication rather than from the onset of microtuberization (this study).

There have been, however, few suggestions as to why the sucrose and maltose could have different effects upon the various plant tissues under *in vitro* conditions.

In biochemical terms the observations are unusual, since sucrose is the major carbohydrate

source supplied to non-photosynthetic plant tissues *in vivo*, and, hence, it would be expected to be the optimum carbohydrate source for supporting growth of plant tissues *in vitro*. Furthermore, when sucrose is supplied to non-photosynthetic plant tissues, it is frequently hydrolyzed by cell wall invertases to glucose and fructose which then enter the cells in which sucrose is metabolized (Lucas & Madore 1988). Hence, glucose and fructose would also be expected to be good carbohydrate sources for supporting growth of cultured plant cells. In contrast, maltose is not known to be supplied to non-photosynthetic plant cells; therefore, it would not be expected to be a better carbohydrate source for the culture of plant tissues than sucrose, glucose or fructose.

The effect of substituting sucrose with maltose or other sugars in a liquid medium on the response of wheat anther culture was investigated (Datta & Wenzel 1987). Maltose instead of sucrose, glucose or fructose in the culture medium led to increase in the number of embryos or plants regenerated from cell cultures. They measured sugar concentrations in the culture media indicating that sucrose was rapidly hydrolyzed much earlier than maltose. From our experiment, a negligible amount of glucose was detected up to harvesting time from the maltose medium by paper chromatography. So it is assumed that extracellular maltose was absorbed through the cell membrane except for a little hydrolysis in the medium during microtuber growth.

3. Invertase activity in culture media

From the time course observation, invertase activity increased during rapid growth of microtubers and then decreased when rapid growth of microtubers stopped (Fig. 6).

Invertase (α -D-fructofuranosidase, E.C. 3.2.1.26) is a highly polymorphic glycoprotein that cleaves sucrose into hexose sugars (Myrback 1960). Investigation of plant tissues has revealed more than one activity of invertase on the basis of pH optima, isoelectric points, solubility characteristics and intra or extra-cellular localization. On the basis of pH optima, plant invertases can be broadly divided into two groups, acid and alkaline enzymes. The soluble alkaline invertase is thought to reside in the cytoplasm (Ricardo & ap Rees 1970). Two types of acid invertases have been described: a soluble (vacuolar) and a particulate form (apoplasmic or cell-wall bound enzyme). The physiological role of acid invertases in

plants is complex and may change in response to developmental (e.g. Strum *et al.* 1995) and environmental signals such as wounding (Matsushita & Uritani 1974; Strum & Chrispels 1990) or gravitopism (Wu *et al.* 1993) and pathogen infection (Strum & Chrispeels 1990; Benhamou *et al.* 1991; Scholes *et al.* 1994; Wright *et al.* 1995; Tang *et al.* 1996).

In the case of cultured cell, the enzyme is secreted into the medium (Thomas *et al.* 1981; Thorpe & Meier 1973; Zamski & Wyse 1985; Masuda *et al.* 1988). Two extracellular invertases were found in the growth medium of the suspension cultures which were probably identical to those in the soluble fraction of callus and seedlings of sugar beet. Extracellular invertase activity in the medium appeared on day 3, when the cells had begun logarithmic growth, and thereafter increased steadily, although its activity was low. These authors suggest that some of the soluble acid invertase passes through the plasma membrane and then some binds to the cell wall, while the rest is secreted into the medium. Furthermore, one of the two invertases, which was found only in the medium, might be secreted directly, and not via the wall, into the medium. Both extracellular invertases had an optimum pH of 5.0, but showed different profiles of activity with changes in pH. Thus, these authors concluded that the invertase found in the medium might be present in a soluble form, and bound to the cell wall, in intact plants.

In the present study, the enzyme detected in the multiplication and tuberization media was of the acid type (Figs. 5 and 62), because the stem and roots submerged in the medium seemed to have invertase with two optimal activities at around pH 4 (Fig. 7). This suggests that there might be a preferential release of acid invertase from the submerged tissues to the medium.

Analysis by SDS-PAGE of crude protein preparations from liquid medium revealed a dominant protein band, which had an apparent molecular mass of 30 kD. Interestingly, invertase from tubers and leaves of potato detected on SDS-PAGE also had a molecular mass of 30 kD (Burch *et al.* 1992). The most satisfactory method to detect invertase isozymes after non-denaturing gel electrophoresis involved the use of an axillary chromogenic system. The gel slabs immersed in soluble staining system overnight showed a single stained band with a pI of 6.75 on the upper side of the gel (Fig. 8). A similar band

appeared to be present in root and stem and in the multiplication medium. A potential biotechnological application from this observation is that the spent tuberization medium or plantlet multiplication medium or potato plantlets *in vitro* could be a source of a particular isozyme of invertase.

Sonneveld *et al.* (1997) found that expression of yeast invertase in the cytosol of potato plant cells led to a reduced tuber size and an increase in tuber number per plant, whereas accumulation of enzyme in the apoplast resulted in a marked increase in tuber size and decrease in tuber number. It was suggested that manipulating sugar levels in various subcellular compartments can have profound effects on sink organ development and that manipulating sugar-metabolism can increase sink size. All have important biotechnological implications.

Sucrose breakdown can occur through two possible pathways in the plant. Sucrose can be broken down into glucose and fructose by invertase (β -D-fructofuranoside fructohydrolase, EC 3.3.3.26). It can also be broken down into fructose and UDP-glucose by sucrose synthase (UDP-D-Glc:D-Fru 2- α -D-glucosyltransferase, EC 2.4.1.13) (Avigad 1982; Morel & ap Rees 1986).

In liquid medium we found accumulation of the same amounts of glucose and fructose while sucrose level was declining, suggesting that invertase, rather than sucrose synthase, was acting on sucrose in the medium during plantlet development or microtuber growth. During the first 3 or 4 weeks, the pH of the plantlet multiplication or microtuberization medium respectively dropped toward being more acidic. This means the medium was more optimal for the acidic invertase activity therein and this was therefore probably the cause of the rapid sucrose hydrolysis at 2-6 weeks.

It can be argued from the time course of invertase activity (Fig. 6) that the initial increase was a response to the presence of sucrose in the medium. When the sucrose disappeared from the medium during tuberization or plantlet growth, the increase in enzyme activity stopped suggesting that the plantlets can produce and/or release the invertase into the medium in response to the sucrose as the required stimulus. Thus the time course of invertase activity correlates with the changes in sucrose status of the medium rather than with plantlet growth or microtuber growth.

4. Relationship between osmotic potential changes in culture media and microtuberization and plantlet multiplication

From the first report (Barker 1953) until recently, microtuberization was studied mostly using sucrose in the medium as an energy source for developing tubers. Few researchers investigated the possible osmotic role played by sucrose. One popular experimental approach has been to substitute other carbohydrates for sucrose, particularly sugar alcohols, in the tuberization medium. Fung *et al.* (1972) cultured etiolated stolon nodes on White's medium (1943) to which sucrose was added at 2, 8 or 12%. The osmotic potentials of the media were made equal using different concentrations of mannitol. Later, it was found that mannitol inhibited microtuberization (Garner 1987).

The use of each of four exogenous sugars or sugar alcohols as an osmoticum (sucrose, glucose, mannitol and sorbitol) is widely accepted in plant tissue culture work, although each is favored for different applications. Glucose is recommended by a number of workers as a very suitable exogenous sugar for routine tissue culture work (Gamborg *et al.* 1975; Michayluk and Kao 1975; Evans *et al.* 1980). Other workers favor sucrose as a exogenous sugar for routine tissue culture (Murashige and Skoog 1962; Shepard and Totten 1977; Bidney and Shepard 1980; Shahin and Shepard 1980). Sucrose alone is favored for regeneration of *Solanum* (Gleddie *et al.* 1983; Mukherjee *et al.* 1991), compared to combinations of glucose and mannitol, or glucose and fructose. Sorbitol was found to be beneficial in culturing meristem tip explants of *Malus robusta* when glucose, sucrose and fructose proved unsuitable (Pua and Chong 1984), although in this case the sugar alcohol was not intended primarily as an osmoticum. It has also been useful in improving apricot culture (Marino *et al.* 1989, 1993). Mannitol is metabolized in some plants (Trip *et al.* 1964; Tholakalabavi *et al.* 1994) and is taken up by potato plants *in vitro* and transported to the shoot (Lipavska and Vreugdenhil 1996); however, for other plants, mannitol is regarded as an inert osmoticum (Oparka and Wright 1988; Wright and Oparka 1990; Do and Cormier 1991).

Khuri & Moorby (1995) reported osmolarity of base medium as 4% sucrose supplemented with additional sucrose (4%), maltose (4% equivalent osmotically to that of sucrose),

glucose (4% equivalent osmotically to that of sucrose) or fructose (4% equivalent osmotically to that of sucrose), with an unsupplemented 4% sucrose as a control. The base medium was one on which *Solanum tuberosum* cv. Estima explants were grown for 12 weeks comprising the plantlet multiplication and *in vitro* tuberization stages. The osmotic potential of all media rose slightly until week 9, but by week 12 it was the same as that at week 1.

Two possible criticisms can be directed again these earlier studies. Firstly, although many attempts were made to adjust the different media to have the same osmotic potential, there was no consideration (except the study of Khuri and Moorby 1995) given to the possibility that the osmolarity of the media might become different during culture. Second, there was no attempt to compare the changes in osmolarity of the media separately during plantlet multiplication and tuberization.

Many researchers suggested that microtuber induction might depend on the osmotic shock of high sucrose solution. In the present study, microtubers were initiated in the presence of a wide range of sucrose concentrations.

Osmotic potential of the standard tuberization medium rose between 2 to 5 weeks before dropping slightly to a steady level until harvest. This pattern of osmotic potential changes seemed to correlate with microtuber growth. However, an examination of the osmotic potential changes in other media investigated here casts doubt on the overall significance of osmotic potential changes in the tuberization medium in relation to microtuber initiation and growth. For example, in the tuberization medium 8% maltose, the pattern of osmotic potential was different from that of the 8% sucrose medium, to the marked increase of microtuber fresh weight within 7 weeks of culture. Osmotic potential changes in multiplication medium was well matched with plantlets biomass increase in the first 4 weeks of culture. It dropped rapidly when that stage was prolonged up to 6 weeks. It was assumed that glucose and fructose were utilized as a carbon source for the plantlet growth after sucrose hydrolysis.

5. Correlation between time course of carbohydrate changes and that of microtuber growth and plantlet growth

Sucrose hydrolysis has been widely reported in cell culture media (Maretzki *et al.* 1974; Thomson & Thorpe 1987; Tremblay & Tremblay 1995) and in media for micropropagation (George 1993).

Here, compositions of sugar in the standard microtuberization medium also exhibited remarkable changes (Figs. 3 and 4) during the rapid phase of microtuber growth (Fig. 2; week 3 to week 6) as did the compositions in the standard multiplication medium (Figs. 60 and 61) during planlet growth (Figs. 57 and 58).

After 6 weeks of culture, sucrose in the tuberization medium was almost completely converted to glucose and fructose, which were present in similar proportions. The cessation of further microtuber growth seems to correlate with the disappearance of sucrose rather than the depletion of carbohydrate from the medium *per se*.

Breakdown of maltose in the medium could be expected to yield two glucose molecules, but the paper chromatographic result showed maltose in the medium remained mostly intact up to 10 weeks, a finding similar to that in the study of Khuri & Moorby (1995). This indicates that there was negligible breakdown of maltose and maltose was utilized via another metabolic pathway to support the development of microtubers.

6. Possible interaction between carbohydrate levels in tuberization medium and gibberellin levels in stolon tips

Phytohormones, particularly gibberellins (GA), have been suggested to play a prominent role in control of tuberization (Ewing 1987; Vreugdenhil and Struik 1989). Exogenous application of GA promotes stolon elongation and inhibits tuber formation (Smith & Rappaport 1969; Kumar & Wareing 1972). It has also been reported that decline of GA activity in potato (*Solanum tuberosum* L.) plants is associated with tuberization (Okazawa 1959, 1960; Smith & Rappaport 1969; Pont Lezica 1970; Railton & Wareing 1973; Krauss & Marschner 1982). Although the inhibiting effect of GA on potato tuberization is well studied, the interaction between GA and other regulating factors on the control of tuberization is still a matter of debate.

Xu *et al.* (1998) reported much higher levels of GA₁ in the tip of stolons growing in media

with 1% sucrose, than with 8% sucrose, suggesting that sucrose could modulate endogenous GA levels in the stolon tip. The significance of this finding lies in another observation that endogenous GA₁ level was high during stolon elongation and remained high under non-tuber-inducing conditions but decreased when stolon tips started to swell under tuber-inducing conditions. Thus GA₁ levels in the stolon tips were negatively correlated with sucrose concentration in the medium indicating that the endogenous GA levels could be closely associated with the sucrose concentration in the medium.

It is possible that sucrose acts as a regulator by influencing endogenous GA levels during tuber formation.

Evidence supporting the role of sucrose as a regulatory signal includes a study increasing the level of sucrose in the stolons by antisense expression of the ADP-Glc pyrophosphorylase cDNA and thus preventing starch formation in the tubers. This led to an increased number of tubers being initiated, even though they did not grow very large (Muller-Rober *et al.* 1992).

The present study the result of varying sucrose levels in the standard tuberization medium is consistent with the idea that different levels of sucrose in the medium could modulate GA levels affecting tuber formation.

Since sucrose in the medium was actually hydrolyzed during tuber initiation and the active phase of microtuber growth (e.g. Fig. 4), the idea that sucrose in the medium could modulate GA levels associated with tuberization should be refined further to include the uptake of glucose and fructose from the medium as being an integral part of the signalling pathway. This is certainly consistent with the results of our experiments of supplying glucose and fructose instead of sucrose in the medium right from the start of the tuberization step (Fig. 19).

7. Effect of medium-replacing treatments

Replacing the medium during microtuberization appeared to have a positive effect on *in vitro* tuberization of both potato cultivars when compared to the standard microtuberization protocol (Figs. 51-55). In both cultivars, it was observed that medium replacement during *in vitro* tuberization tended to favour the production of more larger

microtubers. In particular, more microtubers were formed in the category of 1-2 g when the medium was replaced every 3 weeks, in comparison with the standard microtuberization protocol with one medium lasting for 10 weeks (Figs. 54 and 55). Replacing the multiplication medium did not seem to have a substantial effect on the number of tubers formed in the subsequent standard tuberization protocol (Table 8). However, in both 'Iwa' and 'Daeji' cultivars, thicker and more vigorous shoots resulting from replacing the multiplication medium led to the subsequent formation of larger microtubers when compared to the plants grown in the standard multiplication protocol, i.e. the medium was not changed before transfer to the standard tuberization protocol (Fig. 79). It has been reported that favouring vegetative growth of stolon segments and shoots prior to microtuber induction increases microtuber fresh weight (Chapman 1955; Garner & Blake 1989). There was a slight difference between genotypes as the 'Iwa' plantlets appeared to form heavier microtubers than those of 'Daeji' in response to the standard 8% sucrose-containing tuberization medium regardless of whether the multiplication medium was replaced or not (Figs. 79).

Perhaps, the most remarkable effect of medium replacement during plantlet multiplication on the subsequent *in vitro* tuberization compared to all other treatments in the present study was that some microtubers formed were heavier than 2 g (Figs. 80 and 81). Thus, it seems that there was a carry-over effect of the physiological state of potato plants grown in the multiplication step. Similar carry-over effects have been shown in strawberry (Anderson *et al.* 1982), oil palm (Corley *et al.* 1986) and potato (Satellknecht and Farnsworth 1979) with manipulation of nitrogen level in the medium.

In the present study, the positive influence of medium replacement during plantlet multiplication or *in vitro* tuberization could result from a periodical fluctuation in supply of nutrients, particularly nitrogen and carbohydrates, to the potato plantlets. This might change the endogenous hormonal balance of the plantlets during growth and tuberization.

8. Effect of sucrose concentrations and varying pH in the media on microtuberization

Effect of sucrose concentration

We found no tuber formation in sucrose-free medium, a result similar to the findings of other researchers using different protocols (Xu *et al.* 1998; Lawrence & Barker 1963). Microtubers were induced in media containing sucrose ranging from 2 to 20% (w/v) (Table 3). The number of microtubers formed in this range of 2 to 12% sucrose appeared not to be substantially different (Table 3), but more than 16% sucrose in the medium resulted in a reduction in the number of microtubers.

It was established a long time ago that a high sucrose concentration (5% as opposed to 1%) favoured *in vitro* tuberization of potato from cultured stolon tips of Aram chief potato in Knop's nutrient solution (Mes & Menge 1954). A high sucrose concentration (8% rather than 2%) also seems to enhance microtuber development in the presence of growth regulators in the medium (Harmey *et al.* 1966). On media free of growth regulators, 8% sucrose coupled with transfer to short days promoted microtuber formation (Garner & Blake 1989). Thus the consensus seems to be that a concentration of about 8% sucrose gives better yield of microtubers. The present results confirm that well-grown *in vitro* potato plantlets responded similarly to isolated stolons in culture.

Microtuber size or weight is a more important microtuber character than microtuber number because small sized microtubers are more vulnerable to storage loss (Naik & Sarkar 1997) and are unsuitable for direct field planting (Jones 1988). The production of heavier microtubers could substantially increase yield of potato propagules in the green house and field (Wiersema *et al.* 1987).

The data in the present study on the effect of different sucrose concentrations in the tuberization medium on the average fresh weight of microtubers formed are in general agreement with the studies in the literature using different protocols or different culture systems (Wang & Hu 1982; Hussey & Stacey 1984; Akita & Takayama 1988a,b; Garner & Blake 1989; Leclerc *et al.* 1994; Muller-Rober *et al.* 1990; Akita & Takayama 1994a,b; Akita & Ohta 1998). In both potato varieties, the 8% sucrose medium was different from the other sucrose media because the frequency of microtubers heavier than 1.0 g was observed mainly with the 8% sucrose medium (Figs. 16 and 17).

Effects of varying pH in the tuberization medium

The results in this study showed that microtuber number, hence initiation of *in vitro*

tuberization in 'Iwa', was not influenced by the culturing of plantlets in tuberization media at different pH. In contrast, the data on average microtuber weight seems to indicate a huge genotypic difference between 'Iwa' and 'Daeji', plants in the reaction to the initial pH of the tuberization medium. 'Iwa', unlike 'Daeji', was relatively insensitive to initial pH higher or lower than that of the standard tuberization medium (Figs. 42 and 43). Among all the media variations in this study, pH produced the only this difference between the two cultivars. In the literature, there are many reports on differences among potato cultivars/varieties in response to manipulations of *in vitro* tuberization. Overall our results showed similar trends in responses to the different microtuberization treatments by 'Iwa' and 'Daeji' plantlets.

Few research papers have been published on the influence of medium variations on the weight or size distribution of microtubers formed by well-grown plantlets. Here, the data on the frequencies of microtubers in different weight categories (Figs. 44 and 45) mirrored the result on average microtuber weight showing that the 'Daeji' rather than 'Iwa' plantlets were more sensitive to the high or low pH media resulting in higher frequencies of smaller microtubers being formed in these media.

9. Ultrastructural studies

Green root of *in vitro* potato plantlet

Greening of plant tissue in the light indicates the presence of chloroplasts, which are usually associated with leaf or stem of plants grown in light. No worker has published detailed analysis of the greening of potato roots of plantlets grown *in vitro* and the fate of the green roots of the plantlets during microtuberization which usually took place in darkness for several weeks (e.g. 10 weeks in the present study) has received no prior attention. Since ultrastructural study of green roots of the potato plantlets has not been done and only a limited time was available to complete this thesis, it was decided to initiate a small-scale electron microscopy study of the materials from the 2-step process for microtuber production. Electron microscopy of potato leaf and stem tissues of the 'Iwa' potato plantlets reveals chloroplasts with well developed grana and stroma

thylakoids which are typical of organelles involved in normal photosynthesis. Greening of potato root tissue during the plantlet multiplication step is also due to chloroplast formation therein, but these organelles are more diverse in structure compared with those of the leaf and stem. Chlorophyll formation is dependent on light induction (Castelfranco and Beale 1983), and it must therefore be assumed that the roots of the potato plantlet can respond to light during plantlet growth, otherwise the grana and thylakoid partitions would not have differentiated.

Plant roots usually grow underground as heterotrophic organs, depending on the shoot and leaves for their energy source. Roots may become green when exposed to light or when they develop as adventitious organs (Torrey and Clarkson 1975). In roots of the epiphytic *Ochidaceae* (Benzing *et al.* 1983) and in the aerial roots of mangroves (Gill and Tomlinson 1977), photosynthesis by this organ does, in fact, contribute to the carbon economy of whole plant. Although many roots, *in vivo* or *in vitro*, can become green when grown under light, we do not know to what extent the root, as an organ, has retained its potential for photosynthesis and photoautotrophy. Photoautotrophic green roots of *Acmella oppositifolia* have been maintained *in vitro* for over 2 years (Flores *et al.* 1993). Many *in vitro* root cultures can generate green shoots, suggesting that the root cells remain totipotent (Peterson 1975).

Overall, the chloroplasts found in the green roots after 10 weeks in the dark during the microtuberization step were very similar to those found in green leaves of the plantlets from the plantlet multiplication step. The possibility that the chloroplasts in the roots might contribute to energy or biosynthetic requirement for microtuber formation cannot be ruled out. It is also not clear what would be the influence, if any, that the sucrose in the microtuberization medium might have on the metabolic capacity of the root chloroplasts.

V. Future Studies

1. Time course of changes in major mineral elements and nitrogen in the medium in relationship to the medium replacement treatments will be needed so that we have a more complete picture of the effects of these treatments apart from sucrose addition.
2. The low invertase activity in tuberization medium is possibly because the root-bound surface invertase might be a main source of invertase in medium. So, this enzyme should be extracted and then its changes can be investigated in relation to sucrose utilization, plantlet growth and microtuberization.
3. cDNA clone for invertase can be obtained and will allow a study of the changes in invertase gene expression in tissues submerged in the liquid media during plant propagation and tuberization.
4. The present study can be made more complete if the changes of osmotic potential in the media at various times of the medium replacement treatments involving monosaccharide-containing or the maltose media.
5. This research has led to the formulation of tissue culture media that are suitable not only for potato plantlet multiplication and tuberization but also for relative ease of invertase production and isolation, and recovery of inverted sugars. Some preliminary attempts to purify the acidic invertase from the microtuberization medium were initiated here. When dialyzed ammonium sulfate precipitable fraction (60%w/v) of the tuberization medium was separated using an anion exchange column (1.5x23cm) a broad invertase activity peak resulted and all the fractions had a low level of enzyme activity. Therefore it can be assumed that the enzyme from the medium was not absorbed onto the anion exchanger. Further experiments will be needed to confirm this and develop a more workable enzyme purification scheme.
6. Metabolism of maltose by the potato plantlets will need to be investigated to see how this disaccharide can replace sucrose for microtuberization.
7. The contribution of the green roots of the potato plantlets to microtuberization could be a new fascinating research topic using physiological and biochemical approaches.

VI. Conclusion

Automation of micropropagation and production of microtubers is a final long-term goal that may benefit from this study. For this purpose, production of more bigger microtubers that can compete with traditional seed potatoes is essential.

The standard 2-step process comprising plantlet multiplication and then microtuberization yield bigger microtubers compared to other studies using isolated stolons in culture.

Among the 21 experimental variations to the standard protocols, whether during plantlet multiplication or during *in vitro* tuberization, medium replacement was most effective in inducing the formation of bigger and more microtubers. The treatment of medium replacement during the plantlet multiplication step led to an increase in fresh weight and vigour of the plantlets that would subsequently produce heavier and round-shaped microtubers whereas during tuberization it resulted heavier but longitudinal-shaped microtubers. This manipulation during plantlet growth seems to be more desirable. From the measurement of osmotic potential changes and invertase activity development in the media, it seems that these and possibly other changes in the media may not be strictly related to microtuberization.

VII. References

- Abdullah Z. N. and Ahmad R. (1980) Effect of ABA and GA₃ on tuberization and some chemical constituents of potato. *Plant Cell Physiol.* 21: 1343-1346.
- Addy N. A. (1988) Opportunities and challenges for private industry, *Am. Potato J.* 65: 221-227.
- Aitken-Christie J. (1991) Automation. In: *Micropropagation*, Debergh P. C. and Zimmerman R. H. (eds), Kluwer Academic Publishers, pp. 363-388.
- Akita M. and Ohta Y. (1998) A simple method for mass propagation of potato (*Solanum tuberosum* L.) using a bioreactor without forced aeration. *Plant Cell Rep.* 18: 284-287.
- Akita M. and Takayama S. (1994a) Simulation of potato (*Solanum tuberosum* L.) tuberization by semicontinuous liquid medium surface level control. *Plant Cell Rep.* 13: 184-187.
- Akita M. and Takayama S. (1994b) Induction and development of potato tubers in a jar fermentor. *Plant Cell Tiss. Org. Cult.* 36: 177-182.
- Akita M. and Takayama S. (1988a) Mass production of potato tubers using jar fermentor techniques. *Acta Hort.* 230: 55-61.
- Akita M. and Takayama S. (1988b) Studies of *in vitro* tuberization of potato (*Solanum tuberosum* L.) 4. Effects of hormonal conditions on tuberization. (Abstract.), *Jpn. Soc. Hort. Autumn Meeting* pp. 266-267 (in Japanese).
- Amchem products Inc. (1969) Tech. Serv. Data Sheet E-172. pp. 1-64.
- Anderson H. M., Abbott A. J. and Wiltshire S. (1982) Micropropagation of strawberry plants *in vitro*-effect of growth regulators on incidence of multi-apex abnormality. *Scientia Horticulturae* 16: 331-341.
- Avigad G. (1982) Sucrose and other disaccharides. In: *Encyclopedia of Plant Physiology*, (Loewus F. A. and Tanner W., eds.), Vol. 13, Springer-Verlag, Berlin, pp. 217-347.
- Avila A. de L., Pereyra S. M., Collino D. J. and Argüello J. A. (1994) Effects of nitrogen source on growth and morphogenesis of three micropropagated potato cultivars. *Potato Res.* 37: 161-168.
- Avila A. de L., Pereyra S. M. and Argüello J. A. (1996) Potato micropropagation: Growth of cultivars in solid and liquid media. *Potato Res.* 39: 253-258.
- Bajaj Y. P. S. and Sopory S. K. (1986) Biotechnology of Potato Improvement. In: *Biotechnology in Agriculture and Forestry* (Bajaj, Y. P. S., ed.), Vol. 2, Springer-Verlag, Berlin and Heidelberg, pp. 429-454.
- Barker W. G. (1953) A method for the *in vitro* culturing of potato tubers. *Science* 118: 384.
- Batitus E. J. and Ewing E. E. (1982) Far-reversal of red light effect during long night induction of potato (*Solanum tuberosum* L.) tuberization. *Plant Physiol.* 69: 672-674.
- Batty N. and Dunwell J. M. (1989) Effect of carbohydrate source on the response of potato anthers in culture. *Plant Cell Tiss. Org. Cult.* 18: 221-226.
- Behnke H. (1989) Structure of the phloem. In: *Transport of photoassimilates*. (Barker D. and Milburn J., eds), Longman Scientific, Harlow.
- Benhamou N., Gernier J. and Chrispeels M. J. (1991) Accumulation of β -fructosidase in the cell walls of tomato roots following infection by a fungal wilt pathogen. *Plant Physiol.* 97: 739-750.

- Ben-Hayyim G. and Neumann H. (1983) Stimulatory effect of glycerol on growth and somatic embryogenesis in *Citrus* callus cultures. *Z. Pflanzenphysiol.* 110: 331-337.
- Benzing D. H., Friedman W. E., Peterson G. and Renfrow A. (1983) Shootlessness, velamentous roots and the pre-eminence of *Orchidaceae* in the epiphytic biotope. *Am. J. Bot.* 70: 121-133.
- Bidney D. L. and Shepard J. F. (1980) Colony development from sweet potato petiole protoplasts and mesophyll cell. *Plant Sci. Lett.* 18: 335-342.
- Bizarri M., Borghi L. and Ranalli P. (1995) Effects of activated charcoal effects on induction and development of microtubers in potato (*Solanum tuberosum* L.). *Ann. Appl. Biol.* 127: 175-181.
- Bodlaender K. B. A. (1964) De invloed van groei regulerende stoffen op aardappelen. *Berdrijfsontwikkeling* 3: 596-601.
- Booth A. (1963) The role of growth substances in the development of stolon. *Proc. Easter School Agric. Sci. (Univ. Nottingham)*, 10: 99-113.
- Bottini G. A., Goleniowski M. and Tizio R. (1981) Effect of (2-chloroethyl) Trimethylammonium chloride upon gibberellin levels in potato plants (*Solanum tuberosum* L.) and influence of these phytohormones on tuberization *in vitro*. *Zeitschrift für Pflanzenphysiologie* 103(2): 149-155.
- Bouniols A. (1974) Neoformation de bourgeons *in vitro* à partir de Fragments de racime d'endive (*Cichorium intybus* L.): influence du degré d'hydratation des tissus, et ses conséquences sur la composition en acides aminés. *Plant Sci. letters* 2: 363-371.
- Bradford M. M. (1976) a rapid and sensitive method for the quantative of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Broertjes C. and Harten van A. M. (1978) Application of mutation breeding methods in the improvement of vegetatively propagated crops, An interpretive literature review. *Esevier, Amsterdam*.
- Bronk B. (1975) *Plants consumed by man*. Academic Press, London New York.
- Brown C. L. and Sommer H. E. (1982) Vegetative propagation of dicotyledonous trees. In: *Tissue Culture in Forestry*, (Bonga J. M. and Durzan D. J., eds.), Martinus Nijhoff / Dr W Junk, The Hague, pp. 109-149.
- Bryan J. E., Jackson M. T. and Melendez N. J. (1981) Rapid multiplication techniques for potatoes. *International Potato Center, Lima, Peru*.
- Burch L. R., Davies H. V., Cuthbert E. M., Machray G. C., Hedley P. and Waugh R. (1992) Purification of soluble invertase from potato. *Phytochem.* 31: 1901-1904.
- Büsis D., Heinecke D., Sonnewald U., Willmitzer L., Raschke K. and Heldt H. W. (1997) Solute accumulation and decreased photosynthesis in leaves of potato plants expressing yeast-derived invertase either in the apoplast, vacuole or cytosol. *Planta* 202: 126-136.
- Castelfranco P. A. and Beale S. I. (1983) Chlorophyll biosynthesis, Recent advances and areas of current interest. *Annu. Rev. Plant Physiol.* 34: 241-278.
- Chandra R., Dodds J. H. and Tovar P. (1988) *In vitro* tuberization in potato (*Solanum tuberosum* L.). *Newslett. Intl. Assoc. Plant Tissue Culture*. No. 55: 10-20.
- Chapman H. W. (1955) Potato tissue cultures. *Am. Potato J.* 32: 207-210.
- Chapman J., McGarry A., Carmignac D. F. and Gahan P. D. (1988) Cytochemical

- methods for localization of invertase activity in plant tissues. *Histochemistry* 90: 215-217.
- Claver F. K. (1956) The tuberization of Shoots of potato and callus culture *in vitro*. De la Revista de la Facultad de Agronomía (3a. época), T. XXXII (entregu la), 11-122.
- Claver F. K. (1967) Influence of long day and temperature on tuberization of shoots and plantlets of potato *in vitro*. Revista de Investigaciones Agropecuarias, INTA, Buenos Aires, Rep. Argentina Series 2, Biología y Producción Vegetal, IV (12): 223-230.
- Claver F. K. (1977) Tuberization *in vitro* and culture of potato sprouts contaminated with *Rhizoctonia solani*. Boletín de la Sociedad Argentina de Botánica XVIII, 1-2, 91-96.
- Copeland R. B. (1982) Micropropagation of potatoes. *Agric. Nor. Irel.* 57: 250-253.
- Corley R. H. V., Lee C. H., Law I. and Wong C. Y. (1986) Abnormal flower development in oil palm clones. *The Planter, Kuala Lumpur*, 62: 233-240.
- Courdouroux J. (1967) PhD thesis, Univ. Clermont-Ferrand.
- Daie J. (1984) Characterization of sugar transport in storage tissue of carrot. *J. Am. Soc. Hort. Sci.* 109: 718-722.
- Datta S. K. and Wenzel G. (1987) *Plant Science* 48: 49-54.
- Debergh P. C. (1983) Effects of agar brand and concentration on the tissue culture medium. *Physiol. Plant.* 59: 270-276.
- Deferandez O. S., Martinez L. and Guinazu M. (1995) Qualitative and quantitative production of potato microtubers (*Solanum tuberosum* L.) in two-phase system. *Biocell* 19: 57-63.
- Denton I. R., Westcott R. J. and Ford-Lloyd B. V. (1977). Phenotypic variation of *Solanum tuberosum* L. cv. Dr. McIntosh regenerated directly from shoot-tip culture. *Potato Res.* 20: 131-136.
- De Stecco V. L. and Tizio R. (1982) The action of CCC on tuberization of sprouts of potato tubers cultured *in vitro* in a mineral medium deprived of sugars. *Physiologie Végétale, C R Acad., Sci. Paris Series III*, 294 (10): 901-904.
- Do C.B. and Cormier F. (1991) Accumulation of peonidin 3-glucoside enhanced by osmotic stress in grape (*Vitis vinifera* L.) cell suspension. *Plant Cell Tiss. Org. Cult.* 24: 49-54.
- Dougall D. K. (1981) Media factors affecting growth. *J. Exp. Bot.* 21: 277-280.
- Drew R. L. K. (1979) Effect of activated charcoal on embryogenesis and regeneration of plantlets from suspension cultures of carrots (*Daucus Carota* L.). *Ann. Bot.* 44: 387-389.
- Dunwell J. M. (1985) Anther and ovary culture. In: (Bright S. W. J. and Jones M. G. K. eds.), *Hijhoff ISBN-90-247-3190-9*, pp. 1-44.
- El-Antably H. M. M., Wareing P. F. and Hillman J. (1967) Some physiological response to *d,l*-abscisin (dormin). *Planta* 73: 74-90.
- Escalada M. and de García E. C. (1982) Propagación *in vitro* de *Solanum tuberosum* comométodo para la obtención de plantas libres de virus. *Agronomia Tropical* 21: 91-103.
- Eschrich W. (1980) Free space invertase, its possible role in phloem unloading. *Ber. Dtsch. Bot. Ges.* 93: 363-378.
- Espinoza N., Estrada R., Bryan J. and Dodds J. H. (1984) Tissue culture

- micropropagation, conservation, and export of potato germplasm. Specialized Technology Document, International Potato Center, Lima Peru.
- Estrada R., P. Tovar J. H. and Dodds J. H. (1986) Induction of *in vitro* tubers in a broad range of genotypes. *Plant Cell, Tissue and Organ Culture* 7: 3-10.
- Evans D. A., Sharp W. R. and Flick C. E. (1981) Growth and behavior of cell cultures: embryogenesis and organogenesis. In: *Plant Tissue Culture* (Thorpe T. A. ed.) Academic Press, New York, pp.45.
- Evans D. A., Wetter L. R. and Gamborg O. L. (1980) Somatic hybrid plants of *Nicotiana glauca* and *N. tabacum* obtained by protoplast fusion. *Physiol. Plant.* 48: 225-230.
- Ewing E. E. (1985) Cuttings as simplified models of the potato plant. In: *Potato Physiology*. (P. Li, ed.), Academic Press, Orlando, FL. ISBN 0-12-447660-0, pp. 154-207.
- Ewing E. E. (1987) The role of hormones in potato (*Solanumtuberosum* L.) tuberization. In: *Plant hormone and Their Role in Plant Growth and Development*. (Davies P. J., ed.), Martinus Nijhoff Publisher, Boston, pp. 515-538.
- Ewing E. E. (1990) Induction of tuberization of potato. In: *The molecular and Cellular Biology of the Potato*. (Vayda, M. E. and Park, W. D. eds.), C.A.B. International, Wallingford, pp. 25-41.
- Ewing E. E. (1995) The role of hormones in potato (*Solanum tuberosum* L.) tuberization. In "Plant hormones: physiology, biochemistry and molecular biology" (Davies P. J. ed.), Kluwer, Dordrecht, pp. 698-724.
- Ewing E. E. and Struik P. C. (1992) Tuber formation in potato: induction, initiation and growth. *Hort. Rev.* 14: 89-198.
- Faye L. (1981) A new enzymatic staining method for the detection of radish β -fructosidase in gel electrophoresis. *Analytical Biochemistry* 112: 90-95.
- Finnie S. J., Powell W. and Dyer A. F. (1989) The effect of carbohydrate composition and concentration on anther response in barley. *Plant Breeding* 103: 110-119.
- Flores H. E., Dai Y. R., Cuello J. L., Maldonado-Mendoza I. E. and Loyola-Vargas V. M. (1993) Green roots: photosynthesis and photoautotrophy in an underground plant organ. *Plant Physiol.* 101: 363-371.
- Forsline P. L. and Langille A. R. (1975) Endogenous cytokinins in *Solanum tuberosum* as influenced by photoperiod and temperature. *Physiol. Plant.* 34: 73-75.
- Forti E., Mandolino G. and Ranalli P. (1991) *In vitro* tuber induction: influence of the variety and of the media. *Acta. Hort.* 300: 127-132.
- Fowler M. W. (1978) Regulation of carbohydrate metabolism in cell suspension cultures. In: *Frontiers of Plant Tissue Culture*. Thorpe T. A. (ed.), IAPTC, Calgary, pp. 443-452.
- Fridborg G., Pedersen M., Landström L. E. and Eriksson T. (1978) The effect of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morphogenesis. *Physiol. Plant.* 43: 104-106.
- Fung M. L., Irvine B. R. and Barker W. G. (1972) *In vitro* tuberization of the common potato (*Solanum tuberosum*) is not a response to the osmotic concentration of the medium. *Can. J. Bot.* 50: 603-605.
- Gamborg O. L., Shyluk J. P. and Kartha K. K. (1975) Factors affecting the isolation and callus formation in protoplasts from the shoot apices of *Pisum sativum* L. *Plant Sci.*

Lett. 4: 285-292.

- Garcia-Torres L. and Gomez-Campo C. (1973) *In vitro* tuberization of potato sprouts as affected by ethrel and gibberellic acid. *Potato Res.* 16: 73-79.
- Garner N. (1987) The development and dormancy of microtubers of potato (*Solanum tuberosum* L.) produced *in vitro*. PhD thesis, Wye College, University of London.
- Garner N. and Blake J. (1989) The induction and development of potato microtubers *in vitro* on media free of growth regulating substances. *Ann. Bot.* 63: 663-674.
- George E. F. (1993) Plant propagation by tissue culture. Part 1. The technology. Exegetics Ltd., England.
- Gill A. M. and Tomlinson P. B. (1977) Studies of the growth of red mangrove (*Rhizophora mangle* L.) 4: The adult root system. *Biotropica* 9: 145-155.
- Gleddie S., Keller W. and Setterfield G. (1983) Somatic embryogenesis and plant regeneration from leaf explants and cell suspensions of *Solanum melongena* (egg-plant). *Can. J. Bot.* 61: 656-666.
- Goodwin P. B. (1982) Methods for the rapid propagation of potato. In: *Potato Production in the Humid Tropics*. (L. J. Harmworth, J. A. T. Woodford, and M. E. Marvel, eds.), International Potato Center, Far East and Southeast Asia Regional Office, Los Baños, Laguna, Philippines, pp. 181-196.
- Goodwin P. B., Kim Y. C. and Adisarwanto T. (1980a) Propagation of potato by shoot-tip culture. 1. Shoot multiplication. *Potato Res.* 23: 9-18.
- Goodwin P. B., Kim Y. C. and Adisarwanto T. (1980b) Propagation of potato by shoot-tip culture. 2. Rooting of proliferated shoots. *Potato Res.* 23, 19-24.
- Gopal J. (1996) *In vitro* selection, genetic divergence and cross prediction in potato. PhD. thesis, Punjab Agricultural University, Ludhiana, India.
- Gopal J., Minocha J. L. and Sidhu J. S. (1997) Comparative performance of Potato crops raised from microtubers induced in dark versus microtubers induced in light. *Potato Res.* 40: 407-412.
- Gopal J., Minocha J. L. and Dhaliwal H. S. (1998) Microtuberization in potato (*Solanum tuberosum* L.). *Plant Cell Rep.* 17: 794-798.
- Gregory L. E. (1965) Physiology of tuberization in plants. In: *Encyclopedia of Plant Physiology*. (Ruhland W., ed.), Vol. 15, Springer-Verlag, Berlin, pp. 1328-1354.
- Gregory L. E. (1956) Some factors for the tuberization in the potato plant. *Ann. Bot.* 41: 281-288.
- Hammerschlag F. (1982) Factor influencing *in vitro* multiplication and rooting of the plum rootstock myrabalan (*Prunus cerasifera* Ehrh.). *J. Am. Soc. Hort. Sci.* 107: 44-47.
- Harmey M. A., Crowley M. P. and Clinch P. E. M. (1966) The effect of growth regulators on tuberization of cultured stem pieces of *Solanum tuberosum*. *Eur. Potato J.* 9: 146-157.
- Harvey B. M. R., Crothers S. H., Evans N. E. and Selby C. (1991) The use of growth retardants to improve microtuber formation by potato (*Solanum tuberosum* L.). *Plant Cell Tiss. Org. Cult.* 27: 59-64.
- Hawker J. S. (1985) Sucrose. In: *Biochemistry of Storage Carbohydrates in Green Plants*. (Dey P. M. and Dixon R. A. eds). Academic Press, Orlando, Fl. pp. 1-51.
- Hawkes J. G. (1978) History of potato. In: *The Potato Crop: the scientific basis for improvement*. (Harris P. M., ed), Chapman and Hall, London, pp. 1-69.

- Henshaw G. G. and Roca W. M. (1976) Report of a planning Conference on Exploration and Maintenance of Germplasm Resources. CIP, Lima, Peru.
- Hole C. C. and Dearman J. (1994) Sucrose uptake by the phloem parenchyma of carrot storage root. *J. Exp. Bot.* 45: 7-15.
- Hooley R. (1994) Gibberellins: perception, transduction and responses. *Plant Mol. Biol.* 26: 1529-1555.
- Hooker M. P. (1983) Research for potato in the year 2000. Int. Potato Center, Lima, Peru.
- Hristoforoglu K., Grasl A. and Schmidt J. (1992) Somatic embryogenesis from mature embryos of *Abies alba*. Proc. Int. Conifer Biotechnology Working Group, N. C. State University, Raleigh.
- Hu C. Y. and Wang P. J. (1983) Meristem, shoot tip, and bud culture. In: Handbook of Plant Cell Culture. (Evans D. A., Sharp W. R., Ammirato P. V. and Yamada Y. eds.), Vol. 1., Chapter 5, Macmillan, NY, pp. 177-227.
- Hulscher M., Krijsheld H. T. and Jongedijk E. (1996) Mass propagation of potato tubers in jar fermentors. *Acta Hort.* 440: 533-537.
- Hunter C. P. (1988) In: Plant Regeneration from Microspore-derived Embryoids and Plants of *Hordeum vulgare* L. PhD Thesis, Wye College, University of London.
- Hussey G. and Stacey N. J. (1981) *In vitro* propagation of potato (*Solanum tuberosum* L.). *Ann. Bot. (London)* [N.S.] 48, 787-796.
- Hussey G. and Stacey N. J. (1984) Factors affecting the formation of *in vitro* tubers of potato (*Solanum tuberosum* L.). *Ann. Bot.* 53: 565-578.
- Jackson S. D. and Prat S. (1996) Control of tuberization in potato by gibberellins and phytochrome B. *Physiol. Plant.* 98: 407-412.
- Jackson S. D., Heyer A., Dietz J. and Prat S. (1996) Phytochrome B mediates the photoperiodic control of tuber formation in potato. *Plant J.* 9: 159-166.
- Jolivet E. (1969) Physiologie de la tubérisation. *Annals Physiol. Vég.* 11: 265-301.
- Jones E. D. (1988) A current assessment of *in vitro* culture and other rapid multiplication methods in North America and Europe. *Am. Potato J.* 65: 209-220.
- Jordan M., Montenegro G. and Apablaza G. (1983) Regeneration of PVX-free plantlets of six potato cultivars by shoot-tip culture and Virazole treatment *in vitro*. *Cienc. Invest. Agrar.* 10: 45-52.
- Joyce P. and McCown B. H. (1991) Microtuber propagation of potatoes. US patent No. 5047343.
- Kartha K. K. (1981) Meristem culture and cryopreservation – method and application. In: Plant Tissue culture: Methods and Applications in Agriculture. (Thorpe T. A., ed.), Academic Press, New York, pp. 181-211.
- Kartha K. K. (1982) Cryopreservation of germplasm using meristem and tissue culture. In: Application of Plant Cell and Tissue Culture to Agriculture and Industry. (Tomes D. T., Ellis B. E., Harney P. M., Kasha K. J., and Peterson R. L., eds.), University of Guelph, Guelph, Ontario, Canada, pp. 139-161.
- Keller W. A., Rajhathy F. and Lacapra J. (1975) *In vitro* production of plants from pollen of *Brassica campestris*. *Can J. Gen. Cytol.* 17: 655-666.
- Khuri S. and Moorby J. (1995) Investigations into the role of sucrose in potato cv. Estima microtuber production *in vitro*. *Ann. Bot.* 75: 295-303.
- Kim Y. C. (1982) *In vitro* tuber formation from proliferated shoot of potato (*Solanum*

- tuberosum* L.) as a method of aseptic maintenance. PhD. thesis, Jeon Buk National University, Korea.
- Kochba J. and Spiegel-Roy P. (1973) Effect of culture media on embryoid formation from ovular callus of shamouti orange (*Citrus sinensis*). Z. Pflanzenphysiol. 69: 156.
- Koda Y. and Okazawa Y. (1983) Influences of environmental, hormonal and nutritional factors on potato tuberization *in vitro*. Jap. J. Crop Sci. 52(4): 582-591.
- Konstantinova T. N., Aksenova N. P. and Chailakhyan M. Kh. (1991) Influence of spectral quality of light on reproductive development of *Solanum andigena* under different daylength. Dokl. Akad. Nauk. SSSR 316: 252-255. (In Russian).
- Kostrica P., Polreichova B. and Domkarova J. (1985) The use of *in vitro* tuber formation for the maintenance of potato genetic resources. Genetika a Slechteni 21(4): 269-278.
- Kosuge T. and Conn E. E. (1959) The metabolism of aromatic compounds in higher plants. J. of Biol. Chem. 234, 2133-2137.
- Krauss A. and Marschner H. (1982) Influence of nitrogen nutrition, daylength and temperature on contents of gibberellic and abscisic acid and on tuberization in potato plants. Potato Res. 25: 13-21.
- Kumar D. and Wareing P. F. (1972) Factors controlling stolon development in the potato plant. New Phytol. 71: 639-648.
- Kumar D. and Wareing P. F. (1974) Studies on tuberization of *Solanum andigena*. New Phytol. 73: 833-840.
- Kühn C., Barker L., Bürkle L. and Frommer W. B. (1999) Update on sucrose transport in higher plants. J. Exp. Bot. 50: 935-953.
- Laemli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
- Last D. I. and Bretell R. I. S. (1990) Embryo yield in wheat anther culture is influenced by the choice of sugar in the culture medium. Plant Cell Rep. 9: 14-16.
- Lawrence C. H. and Barker W. G. (1963) A study of tuberization in the potato, *Solanum tuberosum*. Am. J. Potato Res. 40: 349-356.
- Leclerc Y., Donnelly D. J. and Seabrook J. E. A. (1994) Microtuberization of layered shoots and nodal cuttings of potato: The influence of growth regulators and incubating periods. Plant Cell. Tissue and Organ Culture 37: 113-120.
- Lever M. (1973) Colorimetric and fluorometric carbohydrate determination with p-hydroxybenzoic acid hydrazide. Biochemical Medicine, 7: 274-281.
- Levin R., Gaba V., Tal B., Hirsch S. and DeNola D. (1988) Automated plant tissue culture for mass propagation. Bio/Technology 6: 1035-1040.
- Levin R. and Vasil I. K. (1989) Progress in reducing the cost of micropropagation. Newsl. Int. Assoc. Plant Tissue Cult. 59: 2-12.
- Lipavska H. and Vreugdenhil D. (1996) Uptake of mannitol from the media by *in vitro* grown plants. Plant Cell Tiss. Org. Cult. 45: 103-107.
- Loudon P.T., Nelson R. S. and Ingram D. S. (1989) Studies of protoplast culture and plant regeneration from commercial and rapid-cycling *Brassica* species. Plant Cell Tiss. Org. Cult. 19: 213-224.
- Lucas W. J. and Madore M. A. (1988) Sugar uptake in higher plants. In: The Biotechnology of Plants, (Preiss J., ed.), Academic Press, NY, Vol.14, pp. 35-84.
- Macháková I., Konstantinova T. N., Sergeeva L. I., Lozhikova V. N., Golyanovskaya S.

- A., Dudko N. D., Eder J. and Aksenova N. P. (1998) Photoperiodic control of growth, development and phytohormone balance in *Solanum tuberosum*. *Physiol. Plant.* 102: 272-278.
- Makronosov A. T. and Lundina T. N. (1959) On the role of light and dark periods in the photoperiodic reduction of potato –Dokl. Akad. Nauk SSSR 127: 924-927. (in Russian)
- Mangat B. S., Kerson G. and Wallace D. (1984) The effect of 2,4-D on tuberization and starch content of potato tubers produced on stem segments cultured *in vitro*. *Am. Potato J.* 61: 355-361.
- Mares D. J., Marschner H. and Kruss A. (1981) Effect of Gibberellic acid on growth and carbohydrate metabolism of developing tubers of potato (*Solanum tuberosum*). *Physiol. Plant.* 52: 267-274.
- Maretzki A., Thom M and Nickell L. G. (1974) Utilization and metabolism of carbohydrates in cell and callus culture. In: *Tissue Culture and Plant Science* (Street H. E. ed.), Academic Press, London, pp. 329-361.
- Marino G., Magnanini E., Battistini S. and Righetti B. (1989) Effect of hormones and main carbon energy sources on *in vitro* propagation of apricot (*Prunus armeniaca* L.) cvs. 'San Castrese' and 'Portici'. *Acta Hort.* 293: 355-362.
- Marino G., Bertazza G., Magnanini E. and Altan A. D. (1993) Comparative effects of sorbitol and sucrose as main carbon energy sources in micropropagation of apricot. *Plant Cell Tiss. Org. Cult.* 34: 235-244.
- Marschener H., Sattelmacher B. and Bangerth F. (1984) Growth rate of potato tubers and endogenous contents of indolylacetic acid and abscisic acid. *Physiol. Plant.* 52: 16-20.
- Masuda H., Takahashi T. and Sugawara S. (1988) Acid and alkaline invertases in suspension cultures of sugar beet cells. *Plant Physiol.* 86: 312-317.
- Matsushita K. and Uritani I. (1974) Changes in invertase activity of sweet potato in response to wounding and purification and properties of its invertases. *Plant Physiol.* 54: 60-66.
- Mauk S. C. and Langille A. R. (1978) Physiology of tuberization in *Solanum tuberosum* L. *cis*-Zeatin riboside in the potato plants; its identification and changes in endogenous levels as influenced by temperature and photoperiod. *Plant Physiol.* 62: 438-442.
- McCown B. H. and Joyce P. J. (1991) Automated propagation of microtubers of potato. In: *Scale-up and automation in plant propagation*. (Vasil I. K. ed.), Academic Press, San Diego, pp. 95-110.
- Melis R. J. M. and van Staden J. (1984) Tuberization and hormones. *Zeitschrift für Pflanzenphysiologie* 113(3): 271-283.
- Mellor F. C. and Stace-Smith R. (1977) Virus-free potatoes by tissue culture, In: *Applied and Fundamental aspects of plant cell, tissue and organ culture*. (Reinert J. and Bajaj Y. P. S., eds.), Springer, Berlin Heidelberg New York, pp. 616-635.
- Menzel C. M. (1980) Tuberization in potato *Solanum tuberosum* cultivar Sebago at high temperatures: response to gibberellin and growth inhibitors. *Ann. of Bot.* 46: 259-266.
- Menzel C. M. (1983) Tuberization in potato *Solanum tuberosum* cultivar Sebago at high temperatures: response to gibberellin and growth inhibitors. *Ann. Bot.* 46: 259-266.

- Mes M. G. and Mengo I. (1954) Potato shoot and tuber cultures *in vitro*. *Physiol. Plant.* 7: 637-649.
- Michayluk M. R. and Kao K. N. (1975) A comparative study of sugars and sugar alcohols on cell regeneration and sustained cell division in plant protoplasts. *Z. Pflanzenphysiol.* 75: 181-185.
- Mingo-Castel A. M. and Negm F. B., and Smith O. E. (1974) Effect of carbon dioxide and ethylene on tuberization of isolated potato stolons cultured *in vitro*. *Plant Physiol.* 53: 798-801.
- Mingo-Castel A. M., Smith O. E. and Kumamoto J. (1976a) Studies on the carbon dioxide promotion and ethylene inhibition of tuberization in potato explants cultured *in vitro*. *Plant Physiol.* 57: 480-485.
- Mingo-Castel A. M., Young R. E. and Smith O. E. (1976b) Kinetin-induced tuberization of potato *in vitro*: On the mode of action of kinetin. *Plant Cell Physiol.* 17: 557-570.
- Morel G. and Martin, C. (1952) Gguerison de Dahlias atteints d'une maladie a virus. *Comp. Rend. Acad. Sci. Ser. D.* 235: 1324-1325.
- Morel G. (1960) Producing virus-free *Cymbidium*. *Am. Orchid Soc. Bull.* 29: 495-497.
- Morel G. (1965) Clonal propagation of orchids by meristem culture. *Cymbidium Soc. News* 20: 3-11.
- Morel G. (1975) Meristem culture techniques for the long term storage of cultured plants. In: *Crop Genetics Resources for Today and Tomorrow*. (Frankel O. H. and Hawkes J. G., eds), Cambridge Univ. Press. Pp. 327-332.
- Morel S. and ap Rees T. (1986) Sucrose metabolism in developing tubers of *Solanum tuberosum*. *Phytochem.* 25: 1579-1585.
- Morris D. A. and Arthur E.D. (1985) Effect of gibberellic acid on patterns of carbohydrate distribution and acid invertase activity in *Phaseolus vulgaris*. *Physiol. Plant.* 65: 257-262.
- Mukherjee S. K., Rathinasabapathi B. and Gupta N. (1991) Low sugar and osmotic requirements for shoot regeneration from leaf pieces of *Solanum melongena* L. *Plant Cell Tiss. Org. Cult.* 25: 13-16.
- Muller-Rober B. T., Kobmann J., Hannah L. C., Willmitzer L. and Sonnewald U. (1990) One of two different ADP-pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. *Mol. Gen. Genet.* 224: 136-146.
- Muller-Rober B. T., Sonnewald U. and Willmitzer L. (1992) Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. *EMBO J.* 11: 1229-1238.
- Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- Myrback K. (1960) Invertases. In: *The enzymes* (Boyer P. D., Lardy H. and Myrback K. eds.) (2nd edn.). Academic Press, NY, pp. 379-396.
- Naik P. S. and Sakar D. (1997) Influence of light-induced greening on storage of potato microtubers. *Biologia Plantarum* 15: 473-497.
- Nelson R. R. (1984) Strategy for breeding for disease resistance. In: *Crop Breeding. A contemporary basis*. Pergamon Press, Oxford, pp. 32-50.
- Neuman J. (1959) An auxin like action of coumarin. *Science* 129: 1675-1676.

- Neuman J. (1960) The nature of the growth-promoting action of coumarin. *Physiol. Plant.* 13, 328-341.
- Nozeran R. L., Bancilhon-Rossignol L. and Grenan S. (1977) Nouvelles possibilites d'oblertion et de multiplication rapide de clones sains de pomme de terre (*Solanum tuberosum* L.). *C. R. Hebd. Seances Acad. Sci. Paris*, 285: 37-40.
- Nørgard J. V. (1992) Somatic embryogenesis in *Abies nordmanniana* Lk., PhD thesis, Botanic Garden, University of Copenhagen.
- Nørgard J. V. (1997) Somatic embryo maturation and plant regeneration in *Abies nordmanniana* Lk. *Plant Science* 124: 211-221.
- Nørgard J. V. and Krogsrup P. (1991) Cytokinin induced somatic embryogenesis from immature embryos of *Abies nordmanniana* Lk. *Plant Cell Rep.* 9: 509-513.
- Obato-Sasamoto H. and Suzuki H. (1979) Activities of enzymes relating to starch synthesis and endogenous levels of growth regulators during tuberization of isolated potato stolons cultured *in vitro*. *Z. Pflanzenphysiol.* 95: 69-75.
- Obato-Sasamoto H. and Suzuki H. (1979) Activity of enzymes relating to starch synthesis and endogenous levels of growth regulators in potato stolon tips during tuberization. *Physiol. Plant.* 45: 320-324.
- Ohyama A., Ito H., Sato T., Nishimura S., Imai T. and Hira M. (1995) Suppression of acid invertase activity by antisense RNA modifies the sugar composition of tomato fruit. *Plant Cell Physiol.* 36: 369-376.
- Okazawa Y. (1955) Physiological studies on the mechanism of tuberization of potato plants. *Proc. Crop Sci. Jpn.* 23: 247-248.
- Okazawa Y. (1959) Studies on the occurrence of natural gibberellin and its effect on the tuber formation of the potato plants. *Proc. Crop Sci. Soc. Jpn.* 28: 129-133.
- Okazawa Y. (1960) Studies on the relation between the tuber formation of potato and its natural gibberellin content. *Proc. Crop Sci. Soc. Jpn.* 29: 121-124.
- Okazawa Y. (1967) Physiological studies on tuberization of potato plants. *J. Fac. Agric., Hokkaido Uni.* 55: 267-336.
- Okazawa Y. and Chapman H. W. (1962) Regulation of tuber formation in the potato plant. *Physiol. Plant* 15: 413-419.
- Oparka K., Viola R., Wright K. M. and Prior D. A. M. (1992) Sugar transport and metabolism in the tuber. In: Carbon partitioning within and between organisms. (Pollock C. J., Farrar J. F. and Gordon A. J. eds.) BIOS. Scientific Publishers, Oxford, pp 1-26.
- Oparka K. and Wright K. M. (1988) Influence of cell turgor on sucrose partitioning in potato tuber storage tissues. *Planta* 175: 520-526.
- Ortiz-Montiel G. and Losoya-Saldana H. (1987) Potato microtubers: technology validation in Mexico. *Am. Potato J.* 64: 535-544.
- Orshinky B. R., McGregor L. J., Johnson G. I. E., Hucl P. and Kartha K. K. (1990) Improved Embroid induction and green shoot regeneration from wheat anthers cultured in medium with maltose. *Plant Cell Rep.* 9: 365-369.
- Ougham H. J., Jones T. W. A. and Evans M. L. I. (1987) Leaf development in *Lolium temulentum* L.: progressive changes in soluble polypeptide complement and isozymes. *J. Exp. Bot.* 38: 1689-1696.
- Palmer C. E. and Barker W. G. (1972) Changes in enzyme activity during elongation and

- tuberization of stolons of *Solanum tuberosum* L. cultured *in vitro*. Plant and Cell Physiology 13: 681-688.
- Palmer C. E. and Barker W. G. (1973) Influence of ethylene and kinetin on tuberization and enzyme activity in *Solanum tuberosum* L. stolons culture *in vitro*. Ann. Bot. (London) [N.S.] 37: 85-93.
- Palmer C. E. and Smith O. E. (1969) Cytokinins and tuber initiation in potato *Solanum tuberosum* L. Nature (London), 221: 279-280.
- Palmer C. E. and Smith O. E. (1970) Effect of kinetin on tuber formation on isolated stolons of *Solanum tuberosum* L. cultured *in vitro*. Plant cell physiol. 11: 303-314.
- Peterson R. L. (1975) The initiation and development of root buds. In: The Development and Function of Roots. (Torrey J. G. and Clarkson D. T., eds), Academic Press, NY. pp. 125-161.
- Pierik R. L. M. (1990) Cultivo *in vitro* de las plantas superiores. Mundi-Prensa, Madrid, pp. 49-84.
- Plaisted P. H. (1957) Growth of potsto tuber. Plant Physiol. 32: 445-453.
- Pont-Lezica R. F. (1970) Evolution des substances de type gibberellines chez la pomme de terre pendant la tuberisation, en relation avec la longueur du jour et la temperature. Potato Res. 13: 323-331.
- Pratt H. K. and Goelsch J. D. (1969) Physiological roles of ethylene in plants. Ann. Rev. Plant Physiol. 20: 541-584.
- Pressey R. (1969) Potato sucrose synthase: Purification, properties, and changes in activity associated with maturation. Plant Physiol. 44: 759-764.
- Pua E. C. and Chong C. (1984) Requirement for sorbitol (D-glucitol) as carbon source for *in vitro* propagation of *Malus robusta* No. 5. Can. J. Bot. 62: 1545-1549.
- Quak F. (1979) Meristem culture and virus-free plants. In: Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ culture. (J. Reinert and Y. P. S. Bajaj, eds.), Springer-Verlag, Berlin and New York, pp. 598-615.
- Railton I. D. and Wareing P. F. (1973) Effects of daylength on endogenous gibberellins in leaves of *Solanum andigena*. Physiol. Plant. 28: 88-94.
- Ranalli P., Bizarri M., Borghi L. and Mari M. (1994) Genotypic influence on *in vitro* induction, dormancy length, advancing age and agronomical performance of potato microtubers (*Solanum tuberosum* L.). Ann. Appl. Biol. 125: 161-172.
- Ranalli P., Bassi F., Ruaro G., Del Re P., Dicandilo M. and Mandolino G. (1994) Microtuber and minituber production and field performance compared with normal tubers. Potato Res. 37: 383-391.
- Raquin C. (1983) Utilization of different sugars as carbon sources for *in vitro* cultures of *Petunia*. Z. Pflanzenphysiol. 111: 453-457.
- Reisfeld R. A., Lewis U. J. and William D. E. (1962) Disk electrophoresis of basic protein and peptides on polyacrylamide gels. Nature 195: 281-283.
- Ricardo C. P. P. and ap Rees T. (1970) Invertase activity during the development of carrot roots. Phytochem. 9: 239-247.
- Roca W. M., Espinosa, N. O., Roca, M. R. and Bryan, J. E. (1978) A tissue culture method for the rapid propagation of potatoes. Am. Potato J. 55: 691-701.
- Roca W. M., Bryan J. E. and Roca M. R. (1979) Tissue culture for the international transfer of potato genetic resources. Am. Potato J. 56: 1-10.

- Romberger J. A. and Tabor C. (1971) The picea abies shoot apical meristem in culture. I. Agar and autoclaving effects. *Am. J. Bot.* 58: 131-140.
- Rossel G., Bertoldi F. D. de and Tizio R. (1987) *In vitro* mass tuberization as a contribution to potato micropropagation. *Potato Res.* 30: 111-116.
- Sarkar D. and Naik P. S. (1998) Effect of inorganic nitrogen nutrition on cytokinin-induced potato microtuber production *in vitro*. *Potato Res.* 41: 211-217.
- Sattelknecht G. F. and Farnsworth S. (1979) The effect of nitrogen on the coumarin-induced tuberization of potato axillary shoots cultured *in vitro*. *Am. Potato J.* 56: 523-530.
- Sattelmacher B. and Marschner H. (1978) Relation between nitrogen nutrition, cytokinin activity and tuberization in *Solanum tuberosum*. *Physiol. Plant.* 44: 65-68.
- Sawyer R. L. (1979) "Annual Report" International Potato Center (CIP), Lima, Peru.
- Schilde-Rentschler L., Espinoza D. N., Estrada R. and Lizarraga R. (1982) *In vitro* storage and distribution of potato germplasm. 5th International Plant Tissue Culture Congress, (Fujiwara, ed), Japan.
- Schilde R. L., Espinoza D. N. and Estrada R. (1984) Induction of tubers *in vitro* and their utilization for storage and distribution of potato germplasm. In: Abstract of conference paper of the 9th. Triennial conference of the European Association for Potato Research Interlaken, Schweiz, 1-6 July 1984, Switzerland.
- Schloupf R. M., Barringer S. A. and Splittstoesser W. E. (1995) A review of hyperhydricity (Vitrification) in tissue culture. *Quarterly Plant Growth Regulatory Society of America* 23(3): 149-158.
- Scholes J. D., Lee P. J., Horton P. and Lewis D. H. (1994) Invertase: Understanding changes in the photosynthetic and carbohydrate metabolism of barley leaves infected with powdery mildew. *New Phytol.* 126: 213-222.
- Scott P. and Lyne R. L. (1994) The effect of different carbohydrate sources upon the initiation of embryogenesis from barley microspores. *Plant Cell Tiss. Org. Cult.* 36: 129-133.
- Scott P., Lyne R. L. and ap Rees T. (1995) Metabolism of maltose and sucrose by microspores isolated from barley (*Hordeum Vulgare*). *Planta* 197: 435-441.
- Shah N. M. (1945) The chemistry of coumarin. *Chem. Rev.* 36: 1-62.
- Shahin E. A. and Shepard J. F. (1980) Cassava mesophyll protoplasts: isolation, proliferation, and shoot formation. *Plant Sci. Lett.* 17: 459-465.
- Shepard J. F. and Totten R. E. (1977) Mesophyll cell protoplast of potato: Isolation, proliferation, and plant regeneration. *Plant Physiol.* 60: 313-316.
- Singh M. and Verma S. C. (1979) Post-harvest technology and utilization of potato. In: Post-harvest technology and utilization of potato. *Proc. Int. Symp. Simla, New Delhi*, pp. 1-27.
- Singha S. (1982) Influence of agar concentration on *in vitro* shoot proliferation of *Malus* sp. *Almey* and *Pyrus communis* Sackel. *J. Am. Soc. Hort. Sci.* 107: 657-660.
- Skoog F. and Miller C. O. (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp. Soc. Exp. Biol.* 11: 118-131.
- Sluis P. C. and Rivera C. (1984) Microtuberculos. *Agricultura de las Americas Marzo*, pp. 32-33.
- Smith O. E. and Palmer C. E. (1970) Cytokinin-induced tuber formation on stolons of

- Solanum tuberosum*. Physiol. Plant. 23: 599-606.
- Smith O. E. and Rapport L. (1969) Gibberellins, inhibitors, and tuber formation in the potato (*Solanum tuberosum* L.). Am. Potato J. 46: 185-191.
- Solomko E. A. (1965) Useful artificial mutations in potatoes. Genetics. 1: 233-239. (in Russian).
- Sonnewald U., Bauer M., von Schaewen A., Stitt M. and Willmitzer L. (1991) Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuol or apoplast: a powerful tool for studying sucrose metabolism and sink/source interactions. Plant J. 1: 95-106.
- Sonnewald U., Hajirezaei M. -R., Kossmann J., Heyer A., Trethwey R. N. and Willmitzer L. (1997) Increased potato tuber size resulting from apoplastic expression of a yeast invertase. Nature Biotech. 15: 794-797.
- Sopory S. K. (1979) Effect of sucrose, hormones, and metabolic inhibitors on the development of pollen embryoids in anther cultures of dihaploid. Can. J. Bot. 57: 2691-2694.
- Sovari S. and Schieder O. (1987) Influence of sucrose and melibiose on barley anther cultures in starch media. Plant breeding 99: 164-171.
- Stallknecht G. F. (1972) Coumarin-induced tuber formation on excised shoots of *Solanum tuberosum* L. *in vitro*. Plant Physiol. 50: 412-413.
- Stallknecht G. F. and Farnsworth S. (1979) The effect of nitrogen on the coumarin-induced tuberization of potato axillary shoots cultured *in vitro*. Am. Potato J. 56: 523-530.
- Stallknecht G. F. and Farnsworth S. (1982a) General characteristics of coumarin induced tuberization of axillary shoots of *Solanum tuberosum* cultured *in vitro*. Am. Potato J. 59: 17-32.
- Stallknecht G. F. and Farnsworth S. (1982b) Effect of the inhibitors of protein and nucleic acid synthesis on the coumarin induced tuberization and growth of excised axillary shoots of potato sprouts *Solanum tuberosum* cultivar russet burbank cultured *in vitro*. Am. Potato J. 59: 69-76.
- Strickland S. G., Nichol J. W., McCall C. M. and Stuart D. A. (1987) Effect of carbohydrate source on alfalfa somatic embryogenesis. Plant Sci. 48: 113-121.
- Strum A. and Chispeels M. J. (1990) cDNA cloning of carrot extracellular β -fructosidase and its expression in response to wounding and bacterial infection. Plant Cell 2: 1107-1119.
- Strum A., Sebkova V., Lorenz L., Hardegger M., Lienhard S. and Unger C. (1995) Development- and organ-specific expression of genes for sucrose synthase and three isozymes of acid β -fructosidase in carrot. Planta 195: 601-610.
- Swain S. M. and Olszewski N. E. (1996) Genetic analysis of gibberellin signal transduction. Plant Physiol. 112: 11-17.
- Tang X., Ruffner H. -P., Scholes J. D. and Rolfe S. A. (1996) Purification and characterization of soluble invertases from leaves of *Arabidopsis thaliana*. Planta 198: 17-23.
- Thieme R. and Pett B. (1982) Production of tubers *in vitro* and their use in the establishment of a potato store. Arch. Zuechtungsforsch. 12: 257-262.
- Tholalakabavi A., Zwiazek J. J. and Thorpe T. A. (1994) Effect of mannitol and glucose-

- induced osmotic stress on growth, water relations, and solute composition of cell suspension culture of poplar (*Populus deltoides* var. *occidentalis*) in relation to anthocyanin accumulation. *In Vitro Cell Dev. Biol.* 30: 164-170.
- Thomas M., Maretzki A., Komor E. and Sasaki W. S. (1981) Nutrient uptake and accumulation by sugarcane cell culture in relation to the growth cycle. *Plant Cell Tiss. Org. Cult.* 1: 3-14.
- Thomson M. R. and Thorpe T. A. (1987) Metabolic and non-metabolic roles of carbohydrates. In: *Cell and Tissue Culture in Forestry*. (Bonga J. M. and Durzan D. J. eds.), Vol. 1, Martinus Nijhoff Publisher, Dordrecht, pp. 89-112.
- Thorpe T. A. and Meier D. D. (1973) Sucrose metabolism during tobacco callus growth. *Phytochem.* 12: 493-497.
- Tizio R. (1971) The action and probable role of certain gibberellins A-1, A-3, A-4, A-7, A-9, A-13, on stolon growth and tuberization of potato -D. *Potato Res.* 14: 193-204.
- Tizio R. E. and Maneschi E. (1973) Different mechanisms for tuber initiation and dormancy in the potato (*Solanum tuberosum* L.) *Phyton.* 31: (2) 51-62.
- Tizio R. and Goleniowski M. (1985) New evidence with gibberellin nature of the 'root factor' which delays tuberization of potato sprout sections cultured *in vitro*. *Physiologie Végétale*, CR Acad. Science Paris, Serie III, 13: 499-502.
- Torrey J. G. and Clarkson D. T. (1975) *The Development and Function of Roots*. Academic Press, NY.
- Tremblay L. and Tremblay F. M. (1995) Maturation of black spruce somatic embryos: sucrose and resulting osmotic pressure of the medium. *Plant Cell Tiss. Org. Cult.* 42: 39-46.
- Trip P., Krotov G. and Nelson C. D. (1964) Metabolism of mannitol in higher plants. *Am. J. Bot.* 51: 828-835.
- Upadhy M. D., Dayal T. R., Dev B., Chandra V. P., Shadra R. T and Chandra R. (1974) Chemical mutagenesis for day-neutral mutations in potato. In: *Polyploidy and induced mutations in plant breeding*. Proc. Meet FAO/IAEA, Bari (1972) Vienna, pp. 379-383.
- Upham S. (1982) Commercial plant propagation techniques. *Am. Potato J.* 59: 489-490.
- Van den Berg J. H., Vreugdenhil D., Ludford P., Klocek J. and Hendriks T. (1991) Changes in starch, sugar and abscisic acid contents associated with second growth in tubers of potato (*Solanum tuberosum* L.) one-leaf cuttings. *J. Plant Physiol.* 139: 86-89.
- van der Zaag D. E. (1984) Reliability and significance of a simple method of estimating the potential yield of the potato crop. *Potato Res.* 27: 51-73.
- van der Zaag D. E. and Burton W. G. (1978) Potential yield of the crop and its limitations. *EAPR. Surv. Pap.* 7: 7-22.
- Vasil I. K. and Vasil V. (1986) Regeneration in cereal and other grass species, In: *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 3: Plant Regeneration and Genetic Variability. Academic Press, Orlando, FL, pp. 121-150.
- Vecchio V., Andrenelli L., Pagano M. T. and Benedettelli S. (1994) Influence of photoperiod and media culture on potato microtuber production and dormancy (Abstract). *Potato Res.* 37: 440.
- von Schaewen A., Stitt M., Schmidt R., Sonnewald U. and Willmitzer L. (1990)

- Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. *EMBO J.* 9: 3033-3044.
- Vreugdenhil D. and Struik P. C. (1989) An integrated view of the hormonal regulation of tuber formation in potato (*Solanum tuberosum*). *Physiol. Plant.* 75: 525-531.
- Vreugdenhil D. and Helder H. (1992) Hormonal and metabolic control of tuber formation. In: *Progress in plant growth regulation*. (Karssen C. M., Van Loon L. C. and Vreugdenhil D. eds.), Kluwer, Dordrecht, pp. 393-400.
- Wang P. J. (1977) Regeneration of virus-free potato from tissue culture. In: *Plant Tissue Culture and Its Biotechnological Application*. (W. Barz, E. Reinhard, and M. H. Zenk, eds.), pp. 386-391.
- Wang P. J. (1978) Gene preservation and foundation stock seed production of potato *in vitro*. *Proc. IAPIC*, 4th, pp. 105, Abstract.
- Wang P. J., and Hu C. Y. (1982) *In vitro* mass tuberization and virus-free seed potato production in Taiwan. *Am. Potato J.* 59: 33-39.
- Wang P. J. and Hu C. Y. (1985) Potato tissue culture and its applications in agriculture. In "Potato Physiology" (Li, P. H. ed.), pp. 503-577. Academic Press, New York.
- Wareing P. F. and Jennings A. M. V. (1980) The hormonal control of tuberization in potato. In: *Plant Growth Substances*. (Skoog F. ed), Springer-Verlag, Berlin, pp. 293-300.
- Wattimena G. A. (1983) Micropropagation as an alternative technology for potato production in Indonesia. Ph.D. Thesis, University of Wisconsin- Madison Microfilms International, Ann Arbor, Michigan. pp. 202.
- Wattimena G., McCown B. and Weis G. (1983) Comparative field performance of potatoes from microculture. *Am. Potato J.* 60: 27-33.
- Weber H., Borijk L., Heim U., Buchner P. and Wobus U. (1995) Seed coat-associated invertases of fava bean control both unloading and storage functions: cloning of cDNAs and cell-type-specific expression. *Plant Cell* 7: 1835-1846.
- Wenzel G. (1980) The potential and limits of classical genetics in plant breeding. In: *Plant Cell Cultures: Results and Perspectives*. (Scala F., Parisi B., Cella R. and Cifferri I. eds) Elsevier, North Holland Biomed Press, Amsterdam New York, pp. 33-47.
- Wenzel G., Bapat V. A. and Uhrig H. (1983) New strategy to tackle breeding problems of potato. In: *Plant Cell Culture in Crop Improvement*. (Sen S. K. and Giles K. L. eds.), Plenum Press, NY, pp. 337-349.
- Wetherell D. F. and Dougall D. K. (1976) Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. *Physiol. Plant.* 37: 97-103.
- White P. R. (1943) In: *Handbook of Plant Tissue Culture*. Jaques Cattell Press, Lancaster, Pennsylvania.
- Wiersema S. G., Carbello R., Tovar P. and Dodds J. H. (1987) Rapid multiplication by planting into beds microtubers and *in vitro* plants. *Potato Res.* 30: 117-120.
- Williams R. R. (1992) Towards a model of mineral nutrition *in vitro*. In: *Transplant Production Systems*. (Kurata K. and Kozai T. eds), Kluwer Academic, pp. 213-229.
- Williams R. R. (1993) Mineral nutrition *in vitro*-A mechanistic approach. *Austral. J. Bot.* 41: 237-251.

- Williams R. R. (1995) In: The chemical micro-environment. Aitken-Christie J., Kozai T. and Smith M. A. L. (eds), Kluwer Academic, Netherlands, pp 405-439.
- Wright N. S. (1983) Uniformity among virus-free clones of ten potato cultivars. *Am. Potato J.* 40: 381-388.
- Wright D. P., Baldwin B. C., Shepard M. C. and Scholes J. D. (1995) Source-sink relationships in wheat leaves infected with powdery mildew. I. Alterations in carbohydrate metabolism. *Physiol. Mol. Plant Pathol.* 47: 237-253.
- Wright K. M. and Oparka K. J. (1990) Hexose accumulation and turgor sensitive starch synthesis in disc derived from source versus sink potato tubers. *J. Exp. Bot.* 41: 2355-2360.
- Wu L. L., Song I., Karuppiiah N. and Kaufman P. B. (1993) Kinetic induction of oat shoot pulvinus invertase mRNA by gravistimulation and partial cDNA cloning by the polymerase chain reaction. *Plant Mol. Biol.* 21: 1175-1179.
- Xu X., van Lammeren A. A. M., Vermeer E. and Vreugdenhil D. (1998) The role of gibberellin, abscisic acid, and sucrose in the regulation of potato tuber formation *in vitro*. *Plant Physiol.* 117: 575-584.
- Zamski E. and Wyse R. E. (1985) Stereospecificity of glucose carrier in sugar beet suspension cells. *Plant Physiol.* 78: 291-295.
- Zimmerman R. H., Griesbach R. J., Hammerschlag F. A. and Lawson R. H. (1986) In: Tissue culture as a Plant Production System for Horticulture Crops. Martinus Nijhoff, Boston.
- Ziv M. (1991) Vitrication: morphological and physiological disorders of *in vitro* plants. In: Micropropagation. (Debergh P. C. and Zimmerman R. H., eds), Kluwer Academic Publishers, pp. 45-69.
- Zrenner R., Schüler K. and Sonnewald U. (1996) Soluble acid invertase determines the hexose-to-sucrose ratio in cold-stored potato tubers. *Planta* 198: 246-252.

VIII. APENDICES

1. Composition of Murashige & Skoog's Basal Medium

Major salts of MS	mg/l
NH_4NO_3	1650
KNO_3	1900
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
KH_2PO_4	170

Minor salts of MS

KI	0.83
H_3BO_3	6.2
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	22.3
ZnSO_4	8.6
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025

Iron Solutions of MS

Na_2EDTA	37.3
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8

Organics of MS

Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.5
Glycine	2.0

2. Estimation of Glucose: HBH method (Lever 1973)

In this assay an aroylhydrazine reacts with the reducing sugar to form an aroyloszone which forms a coloured chelate with Ca^{++} .
This assay is moderately light sensitive so avoid bright light and store the reaction tubes in the dark.

Reagents

HBH reagent: 50 mM 4-Hydroxybenzoyl hydrazine dissolved in 25 mM sodium citrate containing 10 mM CaCl_2 .

	Compound	50ml	100ml	150ml	200ml	250ml
A	Tri-sodium citrate	0.3675g	0.735g	1.1025g	1.470g	1.8375g
B	Calcium chloride	0.0735g	0.147g	0.2205g	0.294g	0.3675g
C	Sodium hydroxide	0.600g	1.200g	1.800g	2.400g	3.00g
D	Para-hydroxy benzoic acid	0.3805g	0.761g	1.1415g	1.522g	1.9025g
	Distilled water	15ml	30ml	45ml	60ml	75ml

1. Mesure out two volums of distilled water according to the final volume desired.
2. dissolve compounds A and B together.
3. dissolve compounds C and D.
4. combine the two solutions in a suitable volume with distilled water.
5. store at 4°C in the dark.

Glucose standard: 1.0 mM (1.8 mg/ml) glucose in 0.3% benzoic acid.

Procedure

- a. Preparation of calibration curve. Set up a concentration series of tubes containing different amounts of glucose e.g.

Tuber No.	1 (blank)	2	3	4	5	6
1.0 mM glucose	0.0	0.2	0.5	1.0	1.5	2.0
Water	2.0	1.8	1.5	1.0	0.5	0.0

1. Take 20 µl aliquot from each tube and add to 5 ml HBH reagent.
 2. Mix well and heat in boil water bath for exactly 5 min.
 3. Cool and read absorbance at 420 nm (yellow-green filter).
- b. Analysis of samples. Take 20 µl aliquots and add to 5 ml HBH reagent, mix well and proceed as above.

3. Protein concentration determination (Bradford 1976)

Standard Curve

1. From a stock solution (100mg/l) of a reference protein, e.g. bovine serum albumin (BSA in short), prepare several diluted solutions ranging from 0 to 100 µg/ml for protein determination as in step 2.
2. In a test tube, mix the following:
Coomasie blue dye 1 ml
Protein solution 100 µl
Read absorbance at 595 nm.
3. Plot absorbance vs concentration of proteins to obtain a standard curve.

Determination

Determine the protein content in the medium samples collected at the 2 different sampling times.

1. As in step 2 of the procedure to obtain the standard curve, mix 1 ml of the Coomasie blue dye with 100 µl of medium solution and obtain the corresponding absorbance

at 595 nm.

2. Use the standard curve to estimate the protein concentration in the medium solution prepared.

4. Recipes for SDS-PAGE

<u>Separating gel (to make 30ml)</u>	<u>10%</u>	<u>12.5%</u>	<u>15%</u>
Distilled water	8.0ml	5.5ml	3.0ml
1M Tris-HCl, pH 8.8	11.25ml	11.25ml	11.25ml
30% acrylamide:bis solution	10.0ml	12.5ml	15.0ml
10% SDS (w/v)	300µl	300µl	300µl
10% ammonium persulfate (0.1g + 10ml water)	300µl	300µl	300µl
TEMED	15µl	15µl	15µl

Stacking gel

Distilled water	7.5ml
1 M Tris-HCl, pH 6.8	1.25ml
30% acrylamide:bis	1.0ml
10% SDS	100µl
10% ammonium persulfate	40µl
TEMED	15µl

<u>Electrophoresis buffer</u>	<u>1 liter</u>	<u>2 liter</u>
Tris base	4.54g	9.08g
Glycine	21.6g	43.20g
SDS	1.5g	3.00g
Distilled H ₂ O – bring volume to	1 liter	2 liter

<u>Stain (Coomassie brilliant blue)</u>	<u>100ml</u>	<u>500ml</u>
Absolute methanol	50ml	250ml
Acetic acid (glacial)	10ml	50ml
Coomassie brilliant blue	0.1g	0.5g
Distilled water	40ml	200ml

<u>Destain (5% methanol, 10% acetic acid)</u>	<u>1 liter</u>	<u>2 liter</u>
Methanol	50ml	100ml
Acetic acid (glacial)	100ml	200ml

Distilled water	850ml	1700ml
-----------------	-------	--------

<u>Sample buffer</u>	<u>8ml</u>	<u>20ml</u>
Distilled water	3.2ml	8.0ml
1 M Tris-HCl, pH 6.8	1.0ml	2.5ml
Glycerol	1.6ml	4.0ml
10% SDS (w/v)	1.6ml	4.0ml
2-B-mercaptomethanol	0.4ml	1.0ml
0.1% (w/v) bromophenol blue	0.2ml	0.5ml

Add glycerol dropwise, mix then add 2-B-mercaptoethanol dropwise and mix. Aliquot (1ml) into eppendorfs, label and store at 20 °C.

Molecular weight standards

Staining with Coomassie R-250

Dilute concentrate standard 1:20 in sample buffer.

Boil 5 minutes

Label and store at -20 °C.

Load 10µl/well.

Staining with silver stain

Dilute concentrate standard 1:100 in sample buffer.

Boil 5 minutes

Label and store at -20 °C.

Load 10µl/well.

Tris HCl (separating gel) 1M, pH 8.8

	<u>250ml</u>	<u>500ml</u>
Trizma base	30.285g	60.57g

- 1) Dissolve solid in 90% final volume
- 2) pH adjust to 8.8 using conc. HCl

add dropwise approx. volume conc. HCl required for:

500ml	around 8-10ml
250ml	around 4-5ml

- 3) When stable, adjust volume with distilled water, to approximately 5 ml less than final volume
- 4) Check pH and adjust if necessary
- 5) Adjust to final volume with distilled water
- 6) Label and store at 4 °C

Tris HCl (Stacking gel) 1M, pH 6.8

	<u>250ml</u>	<u>500ml</u>
Trizma base	12.114g	30.285g

- 1) dissolve solid in 90% final volume
- 2) pH adjust to 6.8 using conc. HCl
add dropwise approx. volume conc. HCl required for:

100ml	around 8-10ml
250ml	around 20-25ml

- 3) When stable, adjust volume with distilled water to approximately 5ml less than final volume
- 4) Check pH and adjust if necessary
- 5) Adjust to final volume with distilled water
- 6) Label and store at 4 °C

30% Monomer solution (29.2 acrylamide: 0.8 bis)

	<u>100ml</u>	<u>250ml</u>	<u>500ml</u>
acrylamide	29.2g	73g	146g
bis	0.8g	2g	4g

Label and store in a dark bottle at 4 °C

10% S.D.S. (Sodium dodecyl sulfate)

<u>100ml</u>	<u>250ml</u>	<u>500ml</u>
--------------	--------------	--------------

SDS 10g 25g 50g

Label and store at room temperture

5. Silver Stain Procedure

- 1. Soak gel in 50% methanol, 10% glacial acetic acid (200ml) for 30 min. (Can also be left in this solution overnight).
- 2. Wash the twice for 10 min. with distilled (deionized) water.
- 3. Wash in 120 ml of 10% glutaraldehyde (biological grade) for 30 min. (10% glutaraldehyde: 45ml of 25% glutaraldehyde added to 60 ml of distilled water).
- 4. Rinse with 500 ml distilled water for 2hr. with at least 4 times distilled water changes.
- 5. Agitate for 1 hr. with 500 ml distilled water, repeating 3 times, for a total of 4 hr. and drain well. (Can also be washed overnight in 1000 ml distilled water).
- 6. Make the following immediately before use and add in order (in the fume hood):

0.36% (w/v) NaOH (4.5 ml of 1 M/50 ml)	15.3 ml
add dropwise conc. NH ₄ OH (must be fresh saturated solution)	1.03 ml
and slowly, with stirring AgNO ₃ (aq.) 0.2 g/ml	3.0 ml
ethyl alcohol (100%)	55 ml
- 7. Add to the drained gel, soak with agitation for 5 min. (not more than 10 min.), no longer and dispose of this solution down the sink in the fume hood.
- 8. Wash with distilled water for 5 min., repeating 3 times, for total of 20 min. pour off the water.
- 9. Add the following solution

Ethanol	10.0 ml
1% citric acid	5.0 ml
38% formaldehyde	1.0 ml

distilled water to 1 liter
- 10. Remove gel when bands are dark enough, usually 8 to 10 min.
- 11. Place gel in 5% acetic acid to fix and photograph immediately.

6. Native Isoelectric Focusing Gel

To run a native isoelectric focusing gel, the following protocol must be made:

A. Pouring the Gel (pH 4-6)

Native Isoelectric Focusing Gel

5% T (the total monomer concentration, $\%T = (g \text{ acrylamide} + g \text{ bis-acrylamide}) / \text{total volume} \times 100$), 3.3% C (the crossing linking monomer concentration, $\%C = (g \text{ bis-acrylamide}) / (g \text{ acrylamide} + g \text{ bis-acrylamide}) \times 100$). For two 8 x 7 m x 0.75 mm minigels, need to prepare 12 ml.

- 1) 9.7 ml H₂O
- 2) 2 ml Solution A
- 3) 48 µl ampholyte solution pH 3.5-10
- 4) 240 µl ampholyte solution pH 4-6
- 5) 50 µl 10% ammonium persulfate
- 6) 20 µl TEMED

B. Sample Preparation and Application

Native Gel Sample Buffer (2x), 5 ml

- 1) 2ml glycerol
- 2) 200 µl ampholytes (same proportions as for gel)
- 3) 1.8 ml H₂O

1. Mix protein sample with an equal volume of 2x Sample Buffer. Spin 5 min. at 10,000 x g (in eppendorf centrifuge) before applying sample.
2. Load sample into well.

C. Focusing Conditions – to be carried out at room temperature.

7. Desalting

Gel preparation

1. Gradually add dry desalting gel (Bio-Gel P-6DG, Bio-Rad Laboratories, U.S.A.) to

phosphate buffer (pH 6.0) in a beaker. Each gram of dry desalting gel will form approximately 7 ml of packed bed. Use twice as much buffer as the expected packed bed column.

2. Allow gel to hydrate 30 min. at room temperature. Let the gel settle during hydrating.
3. Resuspend the gel in excess buffer. When 90-95% of the gel has settled, remove fine particles by decanting the excess buffer. Adjust to a volume of twice the expected packed bed volume.
4. Degass by aspirating at reduced pressure.

Pouring the column

1. Fix funnel to top of column, close column outlet, and add enough buffer to fill 20% of column.
2. Smoothly pour uniform slurry into column.
3. When 2-5 cm of bed has formed, allow column to flow until fully packed.
4. Remove excess gel and equilibrate column by passing two bed volume of buffer through the column at operating flow rates.

Desalting procedure

1. Drain buffer to the level of gel bed and stop flow. Carefully layer sample onto the bed surface and drain into the bed. Follow this with buffer to wash the sample into the bed. Attach reservoir and begin run.
2. The separation can be monitored by UV-Visible Photometry and the desired fractions collected. Once a column has been characterized, monitoring is no longer necessary since elution volumes will be consistent. The void volume should be 30-35% of the column volume.
3. Wash column with starting buffer until low molecular weight material is completely eluted. Two bed volumes should be sufficient.